RSC Advances

Studies on the physico-chemical characteristics of collagen-pectin composites

Journal:	*RSC Advances*
Manuscript ID:	RA-ART-09-2014-010368.R1
Article Type:	Paper
Date Submitted by the Author:	25-Oct-2014
Complete List of Authors:	Jayakumar, GladstoneChristopher; Central Leather Research Institute, Chemical Laboratory Nagarajan, Usharani; Central Leather Research Institute, CHORD Kawakami, Kohsaku; National Institute for Materials Science, Jonnalagadda, Raghava Rao; CENTRAL LEATHER RESEARCH INSTITUTE, Chemical Laboratory Nair, Balachandran; Central Leather Research Institute, Chemical Laboratory

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

PAPER

Studies on the Physico-Chemical Characteristics of Collagen-Pectin Composites

Gladstone Christopher Jayakumar[a,b] , Nagarajan Usharani[a], Kohsaku Kawakami[b], Jonnalagadda Raghava Rao[a]*, Balachandran Unni Nair[a]

Received (in XXX, XXX) XthXXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

Collagen is a well-known soft tissue utilized as a scaffold material for wound healing, however it is mechanically feeble and prone to rapid degradation in its native state. In order to overcome this, the collagen forms complex with natural or synthetic stabilizing agents for different applications. For diabetic wound dressings, research focuses on cross-linking compounds with anti-glycation activity as they act as a potential therapeutic agent for wound healing. The current study aims to assess whether pectin can be one of the constituent during collagen scaffold preparation. Experimental and theoretical works suggest that pectin can act as a compatible component for collagen in formulating scaffold with an increase in thermal stability of about 14°C as compared to native collagen (62°C). Pectin induces collagen aggregation due to its charged nature and tends to aggregate fast in an ordered way. This helps to combat during excess glycosylation to collagen. There is a minor loss of crystallinity ~~nature~~ and variations in functional groups of collagen due to pectin. The morphological evaluation through SEM and AFM are used to characterize the influence of pectin in structural variations of collagen fibrils. Morphological evaluation confirms there is no significant changes in the fibrillogenesis, however gelling nature of pectin influences uniform film formation. Moreover, Pectin act as an efficient inhibitor for resisting the action of collagenase on collagen and in turn increase its structural stability. Computational studies also attribute to the fact that pectin can act as a suitable ligand for collagen. Moreover, the study confirms that pectin predominantly interact with basic amino acids which act as competitive agent to sugars during the glycosylation process. The current investigation helps to prospect new avenues in collagen tissue engineering applications for diabetic treatment.

1. Introduction

In the current scenario, the application of natural products for diabetic treatment is of major exploration. In spite of the availability of various classes of antidiabetic agents, diabetes remains a challenge worldwide.[1] The prevalence of diabetes mellitus is estimated to ~~be~~increase rapidly and the preponderance of this diabetic population will emerge from developing countries.[2]This has raised a renewed interest in research that investigates the health benefits of natural polysaccharides in the administration of diabetes mellitus. Amid of various natural compounds, pectin is well known for its various medicinal properties which generally compete with glucose for interacting with free available amino groups in proteins by lowering the effective glycation targets in protein of interest.[3]

Pectin is a polyanionic heterogeneous mixture of complex carbohydrates found in the plants.[4] This is supplemented in the diet of laboratory animals as well as human volunteers. This causes lowering of serum and/or liver cholesterol levels.[5] However, the scientific evidence behind the hypocholesterolemic effects of dietary pectin remains indescribable.[6, 7]Understanding, the interactions between pectin and protein is significant in developing satisfactory food texture and biomedical applications.[8] However, due to the number of interactions possible with pectin, there are many opportunities to explore different systems. In the present study, one such polymer composite is observed with pectin.[9]

Collagen is the most abundant protein in vertebrates. Fibrillar collagens are the main component of the Extracellular Matrix (ECM). Along with other ECM macromolecules (e.g. fibronectin and proteoglycans), collagen fibrils provide a framework within which cells may attach and duplicate.[10] These fibrils are characterized by their 67 nm periodic striations.[11] The natural selection of collagen as the molecule of choice for load-bearing tissues and minor changes in the collagen structure during evolution signifies the unique physico-chemical properties.[12,13] These unique characteristics distinguish collagen as an excellent candidate for soft tissue engineering applications. Therefore, gaining control over collagen organization and ultrastructure is of significant importance.[14,15] In the present investigation, the physico-chemical characteristics of collagen-pectin has been

studied extensively. This study has a great significance in the preparation and characterization of collagen scaffold for diabetic wound dressings. Furthermore, in this work it is proposed that pectin as a ligand used to stimulate the binding with receptor collagen to explore the glycosylation mechanism in the treatment of diabetics. Pectin is modified with Gly-X-Y containing peptides through the carbodiimide chemistry and compared with native pectin in promoting protein-carbohydrate interaction. Apart from diabetic treatment measures, the interaction is proposed for other biomedical applications such as cancer treatments (because of its intrinsic properties), gene therapy and as a functionalized biomaterial for tissue regeneration.

2. Materials and Methods

2.1. General

All chemicals are purchased from Sigma Aldrich, India. All chemicals are used without further purification. Ultrapure water is prepared using a water purification system (Milli-Q Biocel A10, Millipore, Billerica, MA, USA).

2.2. Preparation of collagen membranes

Collagen (1 mg/mL) and pectin solution is mixed together and adjusted the pH to 7.4 with phosphate buffer and sodium hydroxide with varied concentration of pectin (0-1% w/w). The resulted mixture poured into a polyethylene sheet to cast membrane. These membranes are used for XRD (X-ray Diffraction), ATR-FTIR (Attentuated Total Reflectance- Fourier Transform Infrared Spectroscopy) and morphology studies.[16]

2.3. SDS-PAGE of pectin interacted collagen

The interaction of pectin with collagen was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by the procedure reported Jayakumar et al. Type I collagen purified from rat tail tendon was used as a standard.

2.4. Differential scanning calorimetry analysis

The thermal properties of the collagen and carbohydrate modified collagen membrane were measured by a TA Instruments DSC-Q2000 Differential Scanning Calorimeter (METTLER TOLEDO Company, Switzerland) in a temperature range from 0 °C to 200°C at a heating rate of 10°C/min.[17]

2.5. Aggregation of collagen-pectin complex

The polydispersity of collagen and collagen-pectin complex in suspension are determined by Dynamic Light Scattering (DLS) equipment (DelsaNano C particle analyser, Beckman counter) with a He-Ne laser (632.8 nm, 35 mW) as light source. The polydispersity and % polydispersity are derived from the Polydispersity index is calculated from a cumulants analysis of the DLS-measured intensity autocorrelation function. In this cumulants analysis, a single particle size mode is assumed and a single exponential fit is applied to the autocorrelation function and the polydispersity describes the width of the assumed Gaussian distribution. All the measurements are repeated three times.[18]

2.6. Turbidity measurements

Collagen is dissolved in 0.05 M acetic acid at 5°C to give a concentration of about 1 mg/ml. The collagen and pectin treated collagen is carried out for determining the fibril formation from the optical rotation at 313 nm in lamda 35 spectropolarimeter. Dilutions stored at 5°C could be used for up to 2 weeks. Solvents are prepared double strength from NaCl and a buffer.[19]

2.7. FTIR analysis

FT-IR studies are carried out for collagen(1 mg/mL) and collagen-pectin at different concentration of pectin (0.2, 0.4, 0.6, 0.8 and 1% (w/w) per mg of collagen)using ATR-FTIR spectrophotometer (Jasco 6200, Japan). All spectra are recorded by absorption mode at 2 cm^{-1} interval and in the wavelength range of 4000–600 cm^{-1} wave numbers.[20]

2.8. X-ray diffraction analysis

X-ray diffraction (XRD) analysis is conducted using a D/Max-2550 PC X-ray diffractometer (Rigaku, Japan) with Cu K a-radiation for collagen membrane prepared with pectin.[21]

2.9.In vitro biodegradation assay

The enzymatic degradation of native collagen and collagen-pectin composites are carried out as reported by Jayakumar et al., 2012.[16] Collagenase treatment is carried out in 0.04 M CaCl2 solution buffered at pH 7.2 with 0.05 M Tris–HCl. The collagen:enzyme ratio was maintained at 50:1. The % collagen degradation is determined by estimating the release of hydroxyproline. This method of determining hydroxyproline involves the oxidation of hydroxyproline to pyrrole-2-carboxylic acid, which complexes with p-dimethylamino benzaldehyde exhibiting maximum absorbance at 557 nm.

2.10. Scanning electron microscopy analysis

The morphologies of the native collagen (1 mg/mL) and collagen-pectin membranes (0.2, 0.4, 0.6, 0.8 and 1% (w/w) per mg of collagen) are observed under a Scanning Electron Microscope (SEM) (SU8000, Hitachi, Tokyo, Japan) at an accelerating voltage of 10 or 15 kV. Prior to scanning under the SEM, the samples are sputter coated for 30 s with platinum coater (E-1030ion sputter, Hitachi, Tokyo, Japan).[22,23]

2.11. Atomic force microscopy examination

Membrane properties of collagen (1 mg/mL) with different concentration of pectin (1% (w/w)) are analyzed by Atomic force microscope (JSPM 5200). Surface topographical characterization is perhaps the most common use of surface probe microscopy techniques.[24]Joel Measurements are performed in the air. For AFM analysis in air, the surface is air-dried in a dust-free enclosure for 24 h and imaged in non-contact mode with a spring constant of about 20–100 N/m and a resonance frequency of about 250–350 kHz. The topography and deflection signals are recorded simultaneously. The deflection set point used, in all the experiments, was optimized to minimise the contact force, i.e. the set point is at the limit for the probe to pull off from the surface; this reduced the damage to the probe that might exert on the sample during this mode of imaging. Prior to the section analysis and measurements, the images are examined by Nanoscope Image Analysis software by using a “height” data type mode. All recorded images are then subjected to standard image processing

involving plane flattening and optimization of the brightness and contrast.

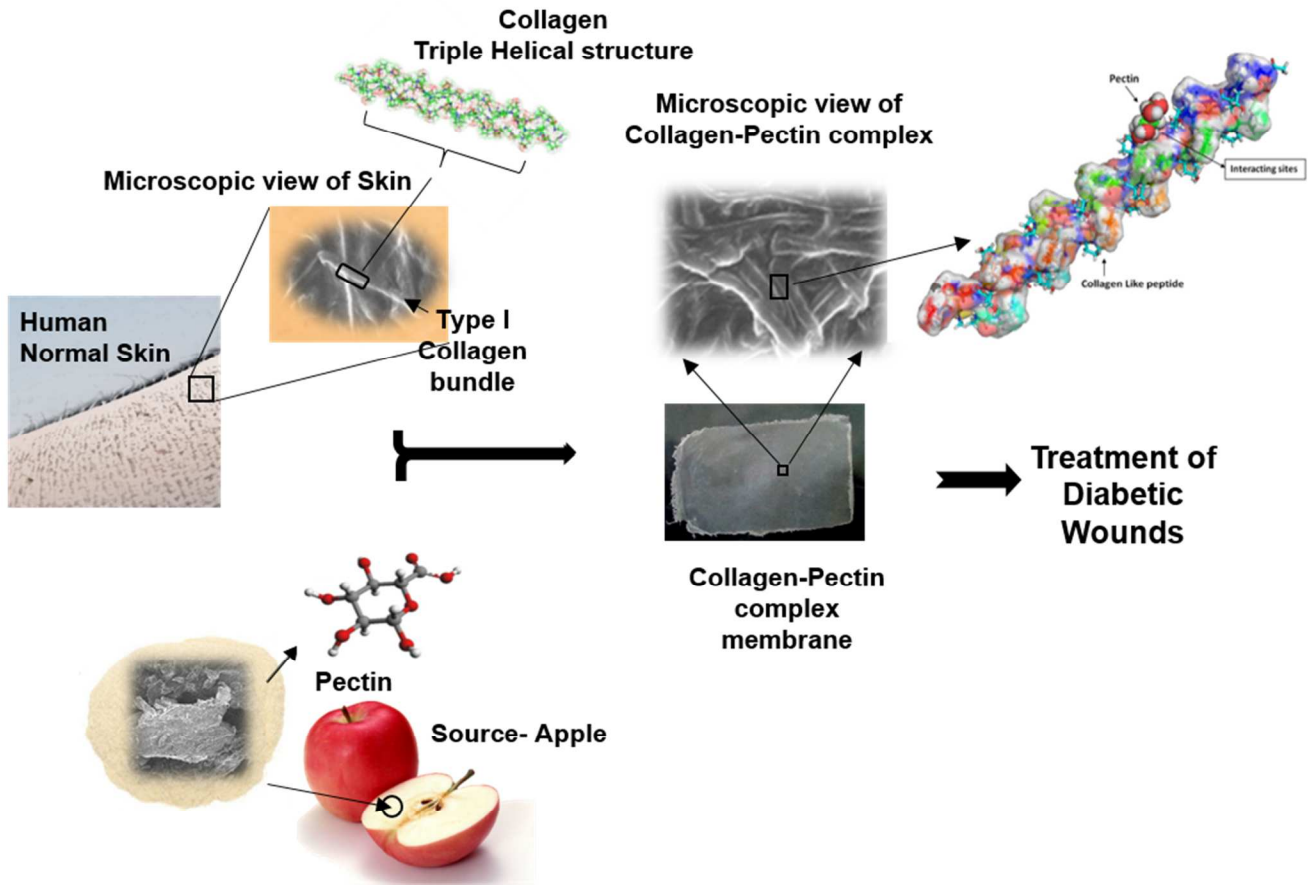
2.12. Computational methods

The biological databases like PubChem, NCBI, PDB (Protein Data Bank), GENCOLLAGEN package (http://www.cgl.ucsf.edu./cgibin/gencollagen.py), Swiss dock (http://www.swissdock.ch/docking), ACDLAB 11.0 and software's like Arguslab and PyMOL are used in the present study. The PDB (Protein Data Bank) is the single worldwide archive of structural data of biological macromolecules, established in Brookhaven National Laboratories (BNL) in 1971 (The Protein Data Bank, 2000). Docking programs have a wide range of applications ranging from protein engineering to drug design.[25]SwissDock, as a web server dedicated to the docking of small molecules on target proteins. It is based on the EADock DSS engine, combined with setup scripts for curating common problems and for preparing both the target protein and the ligand input files. Argus lab offers quite good on-screen molecule-building facilities, with a moderate library of useful molecules. It is a free molecular modelling package that runs under windows.[26] The program reads in molecular coordinate files and interactively displays the molecule on the screen in a variety of representations and color schemes.[27]PyMOL is originally designed to: (a) visualizes multiple conformations of a single structure (trajectories or docked ligand ensembles) (b) interface with external programs, (c) provide professional strength graphics (d) prepare publication quality images, and (e) fit into a tight budget.[28]

In the present study the computational analysis includes the contouring surface of a collagen triple helical structure which holds gaussian sampling over 411 atoms in 0.03 seconds, contoured 52160 triangles (26066 vertices) in 0.06 second and culled 19943 short edges in 6 cycles in 0.19 seconds. While contouring surface for molecule pectin, polar probe and apolar probe with negligible area is used for the analysis.

3. Results and Discussion

The present investigation illustrates about the physico-chemical characteristics of collagen-pectin system. In our earlier studies, pectin has been used a stabilizing agent for the preparation of collagen-pectin microparticles for biotherapeutics. The present works endeavours to find the possible application of pectin as a component for collagen system (Scheme 1). Therefore, to understand the interaction profile of collagen-pectin composite material has been carried out to find the influence of polysaccharides in the various physico-chemical properties of collagen. The properties like functional group and thermal analysis explain the properties of composites. Electrophoretic, X-ray diffraction pattern, turbidity studies explain the order of collagen aggregation induced by pectin. Morphological evaluation of collagen-pectin membrane through SEM and AFM helps to ascertain surface interaction profile. These studies lead to pharmaceutical research to formulate a proper assortment during collagen scaffold preparation for diabetic wound treatment.



Scheme 1 Mechanistic aspects of Collagen–pectin complex

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

PAPER

3.1. Electrophoretic studies

Native collagen and pectin interacted collagen samples are analyzed through electrophoretic studies. The stained SDS-PAGE of the collagen-pectin samples are shown in Fig. 1. Native collagen on L1 displayed one 'α' band (200 kDa) and two 'α' bands (100 kDa for α_1 and α_2), exhibiting polypeptide chains of the triple helix ((α_1 (I)) & (α_2 (I))). The different concentration of pectin (0.2-1%) w/w of collagen is shown in lanes (L2-L6). Pectin interacted collagen on Lane L6 showed higher molecular weight than pure collagen one α_1' band (300 kDa) and two 'α' bands (200 kDa for α_1 and α_2), which is due to unfolding polypeptide chains of the triple helix ((α_1(I))&(α_2(I))). Moreover, electrophoretic studies is carried out to confirm the structural variations in collagen due to the influence of pectin.

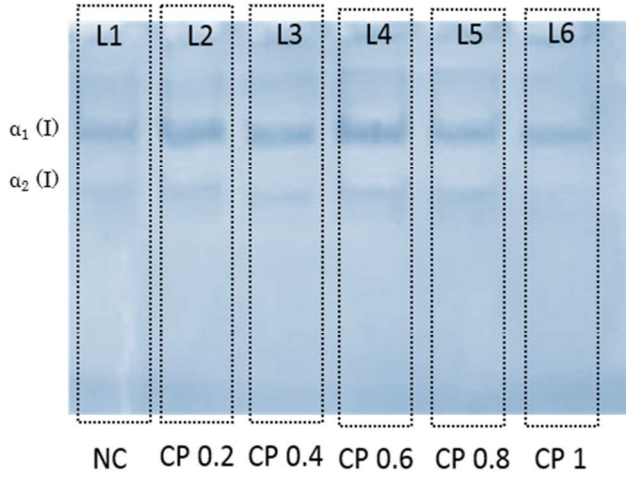


Fig.1. Electrophoretic analysis of collagen-pectin complex at varying concentration of pectin such as 0, 0.2, 0.4, 0.6, 0.8 & 1% (w/w)

3.2. Thermal stability of collagen-pectin complex

Thermal stability of collagen is enhanced by the interaction of pectin. The denaturation temperature of collagen is found to be higher in the carbohydrate mediated collagen. Heat resistance of native collagen found to be 62°C. The different ratios of collagen-pectin of 0.2, 0.4, 0.6, 0.8 and 1 found to be 64, 68, 72, 74 and 76°C respectively. Thermal stability of pectin interacted collagen increased compared to native due to the formation of covalent and hydrogen bond interaction between them. Heat resistance of protein enhanced with increased concentration of pectin. This suggests that collagen molecules are more stabilized in the presence of pectin. Hence, modified collagen is a matrix of homogeneous thermally stable.

3.3. Functional group examination of collagen-pectin complex

FTIR spectra in the range of 4000–600 cm^{-1} of collagen-pectin complex are presented in Fig. 2 (A-F). The major absorption bands of collagen are in the amide band region, including the peak of amide I (1633–1641 cm^{-1}), amide II (1536–1544 cm^{-1}), amide III (1234–1235 cm^{-1}), amide A (3293–3306 cm^{-1}) and amide B (2920–2922 cm^{-1}) as shown in Fig. 2A. Some differences in the FTIR spectra between collagen and pectin mediated collagen are observed in the amide A region. It indicates that collagen might be slightly varied in their secondary structure. Amide I peak of carbohydrate mediated collagen is found at slightly higher wave number as shown in Fig. 2(A-F). This corroborates with the electrophoretic study. The amide I peak is associated with C=O stretching vibration or hydrogen bond coupled with the COO^-. The result suggests that the partial addition of sugars, which might influence the reactive amino acids including lysine, hydroxylysine and histidine for inter and intra-molecular cross-linking localized in collagen molecule. As a consequence of amide I and amide II peak modification is observed in collagen-pectin membrane at 1536–1544 cm^{-1}. This is due to N≡H bending vibration coupled with CN stretching vibration. The amide A peak of modified collagen (3293–3306 cm^{-1}) is associated with the N≡H stretching vibration and the existence of hydrogen bonds. Furthermore, the collagen is mainly stabilized by hydrogen bond as shown by a lower frequency of modified collagen, compared with control collagen. This substantiates the involvement of hydrogen bonding in the collagen-pectin complex. Amide B band of collagen (2920–2922 cm^{-1}) is related with the asymmetrical stretch of CH_2 stretching vibration in the formation of linkage with pectin. The peak corresponding to the amide I peak displays a change as the level of collagen is reduced relative to the concentration of pectin.[29]This indicates the conformational modifications of collagen in the presence of pectin, which is slightly varied and do not involve in instability of the secondary structural backbone.

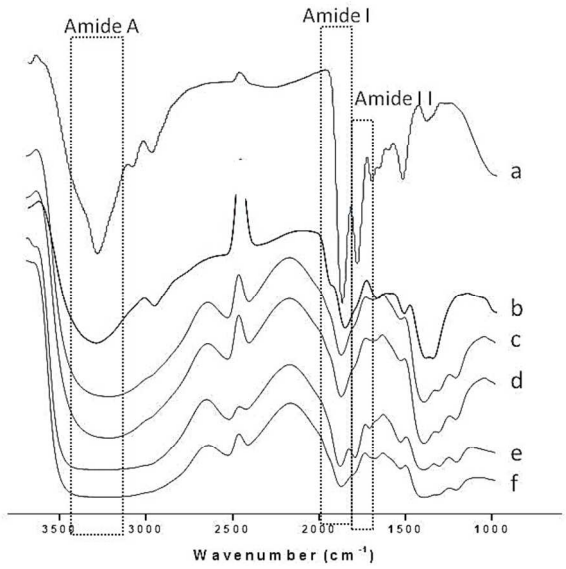


Fig.2. FTIR analysis of collagen (1 mg/mL) with pectin at different concentration such as 0 (A), 0.2 (B), 0.4 (C), 0.6 (D), 0.8 (E) and 1 (F)% (w/w)

3.4. Polydispersity of collagen-pectin complex

Batch mode-Dynamic light scattering (DLS) is a widely-used simple and fast method to determine the size distribution of collagen-pectin colloids. During DLS measurement, laser light (typically 632.8 nm) is scattered by particles in solution. By measuring the fluctuations of the scattered light, induced by Brownian motion (random diffusion) of the particles in the laser beam, the diffusion coefficient is determined mathematically. The poly dispersity index (PDI) value of the native collagen and collagen-pectin complex is indicated in Fig. 3. As the concentration of pectin increases the PDI value of the collagen-pectin complexes founds to be increasing that of native collagen. Moreover, there is increase in polydispersity with increase in pectin concentration, which is due to the increase in the number of cluster points (points of interaction between aggregates or fibrils of collagen, which includes collectively those of hydrogen bonding, electrostatic, hydrophobic natures, etc.) per volume.[30] Here, the high polydispersity index refers to the presence of more heterogeneous particles. The increase in interaction sites of pectin resulted in the limited movement of collagen molecules. Therefore, it reflects the reduction in the mobility of clusters of collagen aggregates.

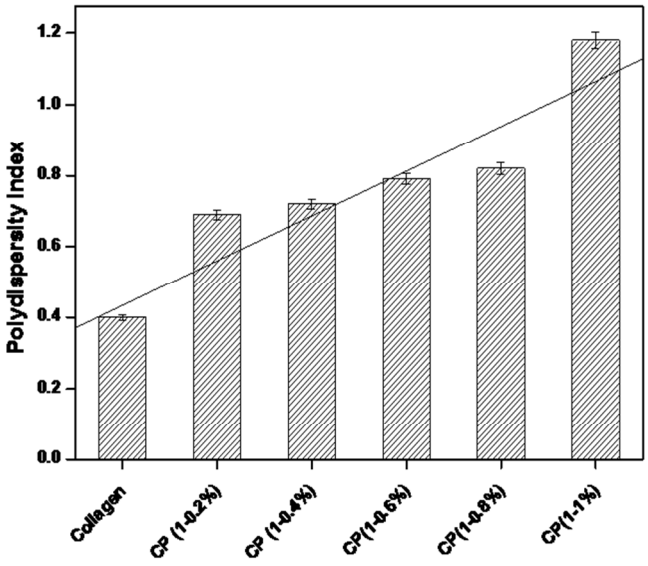


Fig.3. Polydispersity index value of collagen (mg/mL)–pectin complexes at different ratios 0, 0.2, 0.4, 0.6, 0.8 and 1% (w/w)

3.5. Fibrillogenesis of collagen-pectin complex

The formation of collagen fibrils in the ordered form of precipitates in which it is supersaturated under the particular conditions is shown in Fig. 4. The general fibril formation of collagen depends mainly on aggregation of particles in solution.[31,32] The mechanism of precipitation is probably a better way to understand the influence of external stabilizing agent in the collagen.[33]

This is likely to be in the formation of collagen fibrils, where highly asymmetric soluble particles aggregate in a very specific manner with their axes parallel to the ultimate fibril axis.[34,35] Carboxylic groups of pectin greatly influence the fibril formation of collagen in higher rates. In the presence of pectin, collagen aggregation (time dependent) increased with an increase in the concentration. This confirms that the presence of pectin does not affect the order of fibril formation (Fig. 4). Fibrillogenesis of collagen with pectin provides a rational way in understanding the mechanism of treating diabetic wounds.

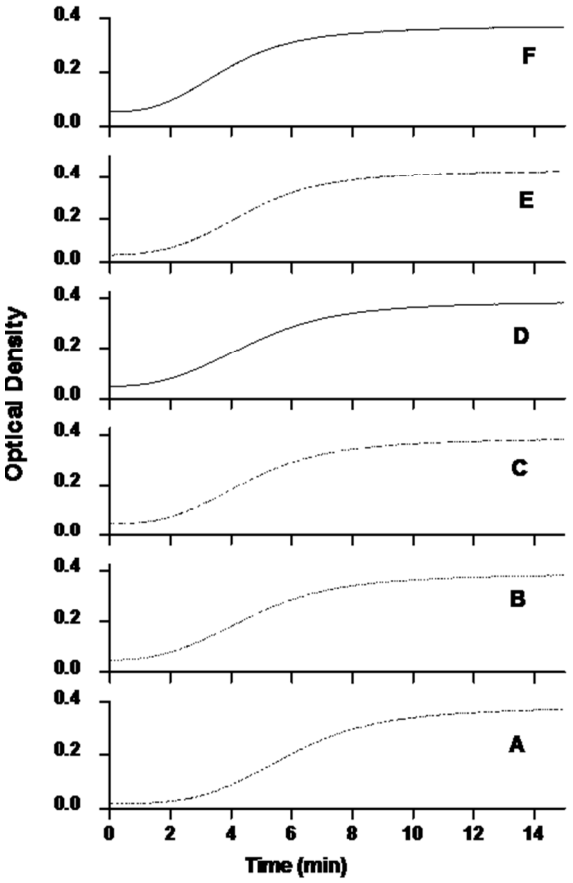


Fig. 4. Turbidity measurements for collagen–pectin complexes at different ratios (1- 0 (A), 1-0.2 (B), 1-0.4(C), 1- 0.6(D), 1-0.8(E) and 1-1(F) %)

3.6. X-ray diffraction pattern of collagen-pectin complex

X-ray diffraction is used to investigate the collagen fibril distribution and orientation in mineralized tissues. Fig. 5 showed the X-ray spectrum of the collagen-pectin complex at varying concentration of pectin. There are two diffraction peaks at the diffraction angles (2θ) for native collagen about 7.44° and 19.78°. The first one is sharp, but the second one is wide, which are in accordance with the characteristic diffraction peaks of collagen.[37] From the Bragg equation d(Å) = λ/2sinθ (λ = 1.54 Å), the minimum values (d) of the repeat spacing's are calculated. The d of the sharp peak was 11.87 Å and that of the wide peak is 4.48 Å, which are related to the diameter of the triple helix collagen molecule and the single left-hand helix chain.[38] With respect to the native collagen, collagen–pectin complex retains the similar helical structure in the presence of pectin. With increasing concentration of pectin sharp peak disappears and the wide peak remains even wider as shown in Fig. 5. This indicates the loss of crystalline nature and acquiring the amorphicity behavior in collagen due to pectin.

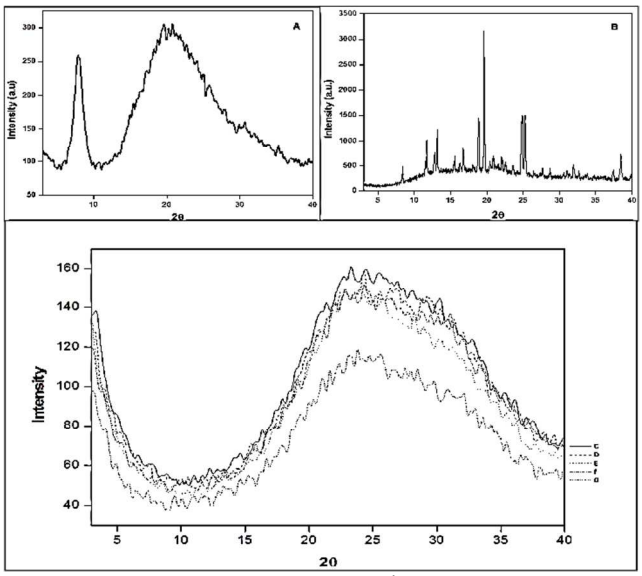


Fig. 5. Diffraction pattern for collagen (1mg/mL) (A), pectin (1%) (B) and collagen-pectin complexes (1-0.2% (C), 1-0.4% (D), 1-0.6 %(E), 1-0.8 %(F) and 1-1% (G))

3.7. Effect of enzymatic resistance of collagen during pectin treatment

Collagenase resistance is carried out for the collagen-pectin composite to understand the enzymatic resistance. Usually, any crosslinking or stabilizing agent imparts heat and enzyme resistance. Pectin has a tendency to react with amino and carboxyl groups which usually renders higher stability to protein. However, the same functionality might interact with collagenase and reduce the activity. Native collagen showed about 5% collagenase resistance. In the presence of pectin collagenase resistance found to be more and higher with increase in concentration of pectin. It is a known fact that pectin can be easily degradable by pectinase and resistance to other enzymes. In collagen-pectin composites, the collagenase resistance found to be 14, 26, 35, 48 and 60% for 0.2, 0.4, 0.6, 0.8 and 1(w/w pectin/mg of collagen) ratio respectively. The resistance collagenase digestion was increased by pectin treatment. These results indicate that collagen structural modifications promoted by pectin are less drastic compared to native.

3.8. Morphological evaluation of collagen-pectin membrane

During *in vitro* condition, increasing the temperature of a neutralized solution of acid (telocollagen) or pepsin extracted type I collagen molecules results in the self-assembly of the monomeric collagen molecules into an isotropic fibrillar network (Fig. 6 (A-F)). The self-assembly of collagen in the presence of pectin spontaneously continues by end-to-end and lateral fusion of molecules induced by the charged nature of polysaccharide. During fibril formation, it is essential that individual collagen units are arranged in such a way that their polarity is parallel, which aids in fibrillar network formation. Consequently, self-assembly of the collagen molecules will result in the formation of microfibrils. Usually, the process of collagen self-assembly is entropy driven process.[39] Hence, this indicates that collagen fibril formation in the presence of pectin is an endothermic process. However, when fibril assembly occurs, collagen molecules with pectin are confined in a tight space which results in the decrease

of the molecule's entropy. Therefore, during self-assembly of the collagen–pectin execute into a disorganized fibrillar network Fig. 6 (B-F). The chemical bond between collagen molecules with pectin inside a protofibrils is in the form of a covalent bond, making them very stable structures[40]. In contrast, the chemical bonds between protofibrils within a fibril are less stable and are reversible (e.g. hydrogen and electrostatic bonds).

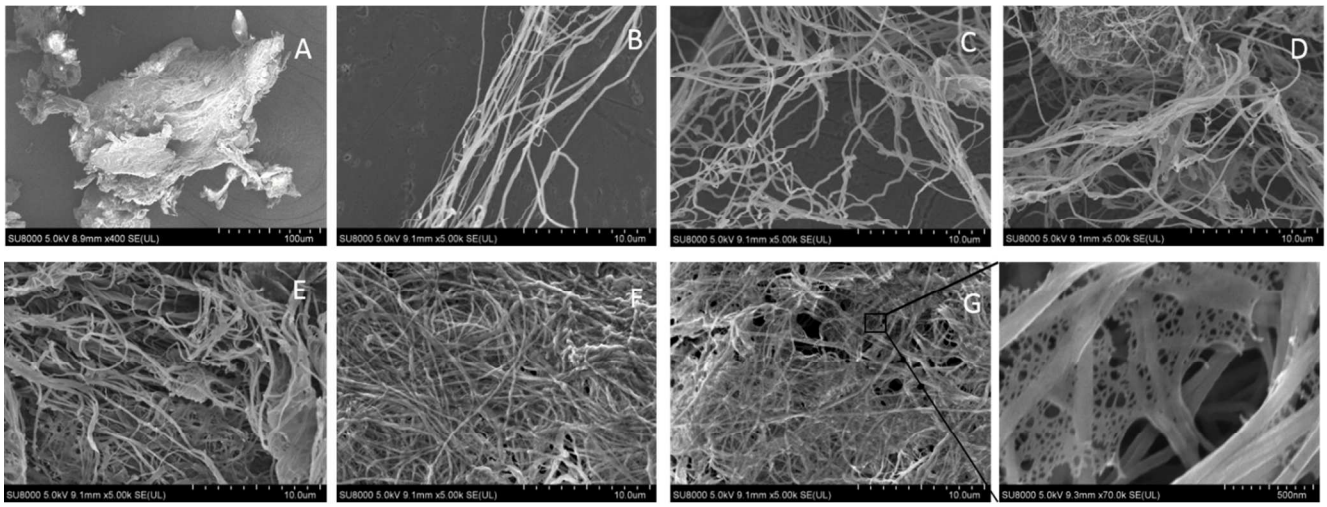


Fig. 6. SEM analysis for pectin (A), collagen (B) and collagen-pectin complexes (1-0.2% (C), (1-0.4% (D), 1-0.6% (E), 1-0.8% (F) and 1-1% (G))

3.9. AFM imaging

To appreciate the influence of pectin in structural variations of collagen is ascertained with AFM images. Collagen is uniquely signified with D banding patterns as seen in Fig. 7A. Alteration in the D pattern may stabilize or destabilize the collagen. The native collagen molecules with pectin possess the tendency to form linear structures with fibril arrangements.

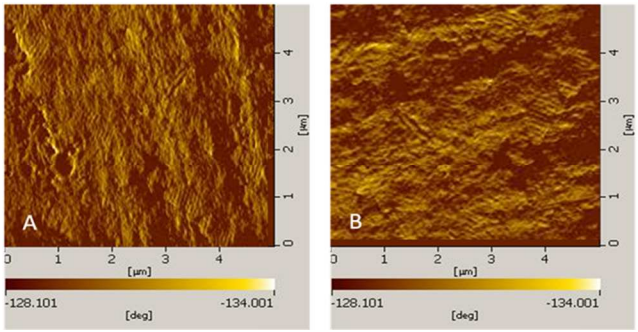


Fig. 7. AFM imaging of native collagen (A) and collagen-pectin complex 1:1(B)

In the presence of pectin, collagen aggregates in the presence of pectin at higher concentration (1:1:: Collagen: Pectin (w/w)) which shows a prominent light and dark patches that are barely visible in the image. AFM images of native collagen and collagen-pectin complex is shown in Fig. 7 (A-B) respectively. Using AFM, the molding technique for the collagen-pectin complex was employed to obtain an imprint that has the same topography of collagen fibrils. The homogenous density, possibly varies among the complexes.[41]The AFM image of pectin reconstituted collagen describes the axial arrangement of collagen-pectin molecules similar to the native fibril form.

3.10. Computational analysis of collagen-pectin complex

The structure of pectin (Fig.8A) is obtained from Pubchem compound search, optimized and then the surface is contoured for the docking studies and it is given in 1. The structure possesses a primary surface area of about 162.96 Å and measuring volume of about 156.16 respectively. Culling process reduced surface complexity by 75 per cent (492 triangles, 248 vertices). Total contouring time for pectin surfaces included 0.02 seconds.

Table 1 The contouring structural parameters for collagen like peptide and pectin molecule

Parameters	Collagen like peptide	Pectin
Gnomonic Projection	0.05 s	0.03 s
Min, Max, Ave Triangles	0.03, 16.89, 0.86	0.01, 0.13, 0.04
Typical edge arc	4.62º	4.62º
Typical edge length	0.74Å	0.27 Å
Average radius	9.17 Å	3.35 Å
Maximum radius	38.24 Å	5.22 Å
Surface area	3856.14 Å^2	187.33 Å^2
Radial Surface Area	1486.21	146.89

Total contouring time for collagen like peptide consumed about 0.30 seconds. The surface traversal process originates 10 surface segments in 0.02 seconds. It resulted in the area for the primary surface (3930.81) and secondary surface (64.17) along with volume of primary surface (6385.25) and secondary surface (8.15). It culled 2 small segments in 0.03 seconds. Culling process reduced surface complexity by 76 per cent (12222 triangles, 6111 vertices). For the pectin surface gaussian sampling time for about 13 atoms is 0.0 s. It contoured 2028 triangles (1016 vertices) in 0.02 seconds. Culled 768 short edges in 5 cycles (430, 202, 100, 30 and 6) are identified. During the process of surface traversal it is identified with 1 surface

segment.

The collagen triple helix (24-mer) is built using Object Technology Framework (OTF) using the GEN COLLAGEN package (http://www.cgl.ucsf.edu./cgibin/gencollagen.py). 24-mer triple helix is minimized with a dielectric constant of 4.0 as given in Fig. 8B. The energy minimisation of triple helix geometry is minimised using a steepest decent method followed by conjugate gradient algorithm. The sequences of the triple helical structure are given as follows

(Gly-Pro-Ile-Gly-Pro-Val-Gly-Pro-Ala-Gly-Ala-Arg-Gly-Pro-Ala-Gly-Pro-Gln-Gly-Pro-Arg-Gly-Pro-Hyp) α_1
(Gly-Pro-Ile-Gly-Pro-Val-Gly-Pro-Ala-Gly-Ala-Arg-Gly-Pro-Ala-Gly-Pro-Gln-Gly-Pro-Arg-Gly-Pro-Hyp)α_1
(Gly-Ala-Val-Gly-Pro-Ala-Gly-Ala-Val-Gly-Pro-Arg-Gly-Pro-Ser-Gly-Pro-Gln-Gly-Ile-Arg-Gly-Pro-Hyp)α_2

The collagen like peptide and pectin are positioned by docking mechanisms using hex software. Here, during docking 1 pair of starting orientations is performed using the working buffer for 1000000 orientations. In the orientations the starting energy (E_{start}) measured about -1.96 KJ/mol with the energy of electrostatics (E_{shape}) of about -1.96 respectively. Correspondingly, the simulations resulted in the measurement of energy minima (E_{min}) -158.59 KJ/mol and energy maxima (E_{max}) -39.39 KJ/mol. The orientations of the docking included parameters such as time scan at 165.52 s, generating functions (GF) at 97.99 s, first Fourier transforms (FFT) at 307.42 s, Scan rate of 27.10 s and FFT Rate of 10321349/s. The Docked structures of collagen like peptide-Pectin complex with a total of 17 min and 1 sec as shown in Fig. 8C. The data obtained correlates the presence of interaction moieties between collagen and pectin and explains towards the prediction of protein interaction partners using physical docking. The docking correlation of these complexes is obtained by its RMS deviation and steric clashes. Clustering methods obtained 11 clusters from 2000 docking solutions is obtained. Beyond correlations it is found that docking detected about orientations with main-chain bumps. Moreover, Molecular mechanics used 2000 energy evaluations (811.69 energies/s) during docking orientations. It is observed that the clustering analysis and docking studies portrayed the binding preferential sites for pectin on the collagen like triple helix molecule. The interaction studies between collagen like peptide and pectin obtained by computational analysis describes the data based on experimental parameters. It is also noted that collagen and pectin are placed at an intermolecular distance of 33.47Å with setting distance range of about 14.25 to 53.25Å using steps of about 0.75. Electrostatics for collagen-pectin complex is calculated for about 315 atoms in 0.01 seconds.

Molecular docking results are used to identify the lowest RMSDs between the docked structure and the crystallographic structure. These results, obtained by docking simulations, strongly indicates that it's a good way to predict the binding pocket of pectin in collagen. In this study, 100 docked conformations of collagen-pectin represent 100 grip points on the complexation energy surface. It has been proposed that collagen-pectin complexation finds pectin as the binding pocket on, the protein and bind in a distinctive way.

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

PAPER

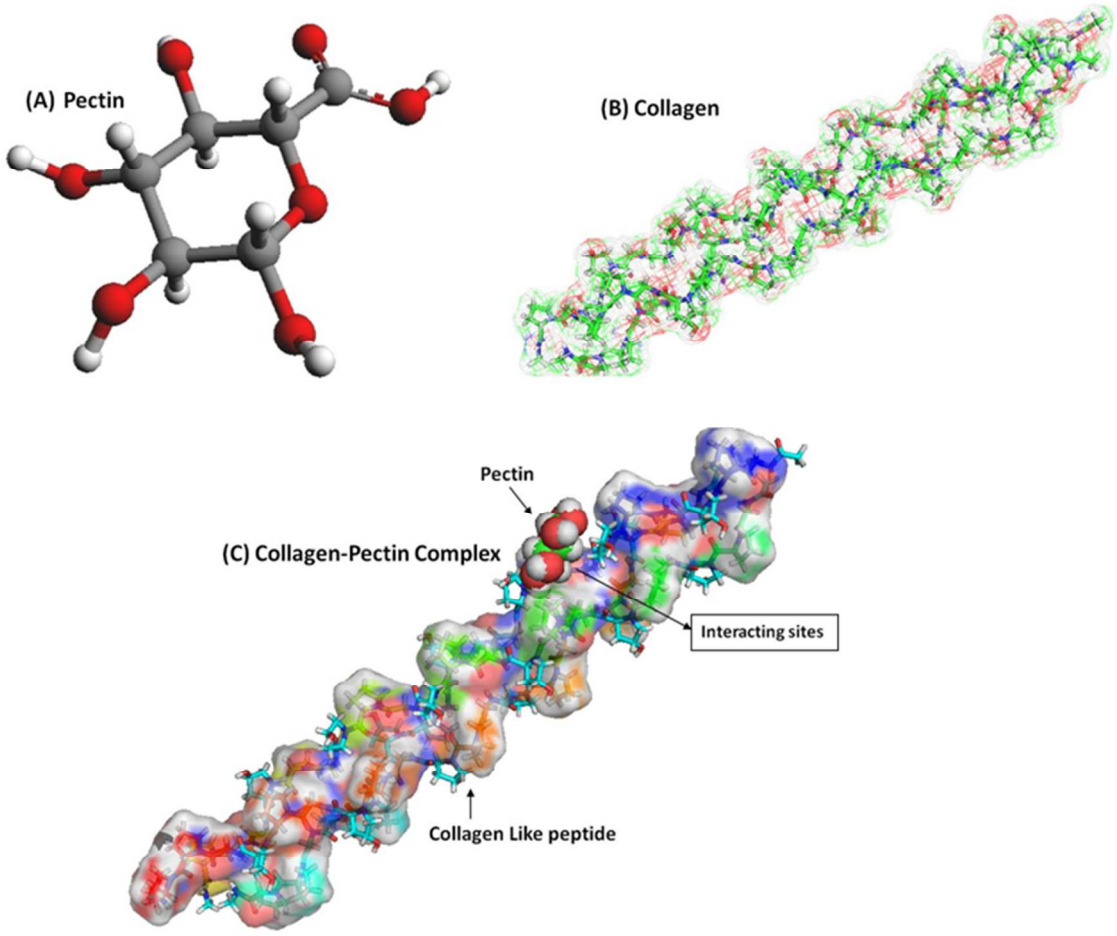


Fig. 8. Energy minimized docked structures of ligand pectin (A), receptor collagen (B) and receptor-ligand docked structure of collagen-pectin complex (C)

3.10. Collagen-pectin interaction profile for soft tissue engineering applications

Collagen and pectin are ~~a~~ two class of compounds which finds impeccable applications in the field of biotherapeutics.[41] Collagen is a natural gift with unique properties like self-healing ability and physical strength. However, the major negative properties are easily prone to enzymatic attack and denaturation occurs when it is subjected to higher temperature. Most of the biomaterial applications highly demand enzyme and heat resistance. On the other hand, pectin is known as the main component in food industry owing to its gelling and emulsifier properties. As compared to well-known natural polysaccharides like chitosan, pectin is readily soluble and imprint immense benefits for specific modifications. Therefore, the necessity of understanding the interaction profile between collagen and pectin is inevitable in the perpetual of soft materials. Physico-chemical properties is always analyzed for any biomaterial composites to understand the efficiency of stabilizing or crosslinking agents. Similarly, in the current investigation, techniques like electrophoretic, thermal stability, aggregation profile, fibrillogenesis, functional groups modifications, conformational changes, morphological assessment, collagenase resistance and *in silico* approaches are carried out to understand the composite profile. Collagen is a three dimensional matrix with three polypeptide chains, usually heterotrimeric. The presence of these bands is visually stained using SDS-PAGE. Any modifications in the native collagen can be visually ascertained through this technique. This is probably due to the interaction of pectin with collagen, which result in single or two bands. The main advantage of pectin is solubility and hydrophilic nature. This perpetuates convenient interaction with collagen. Moreover, pectin are expected to interact with collagen in three different modes, such as hydrogen, covalent and weak ionic interactions. From the SDS-PAGE results, it is attributed that pectin can

interacts with collagen. Consequently, thermal resistance is an serious concern in the context of biomaterials. This is mainly depends on the specific end applications. However, pectin is a challenging candidate, which incorporate thermal resistance to the native collagen. Functional groups are considered to be a signature in the biological materials as it is accountable for its functional annotations. Collagen unique signatures are the presence of amide bands when analyzed through FT-IR. It is presumed that some significant changes in the functional groups will directly impact the functionality of the compounds. Therefore, collagen-pectin composite is analyzed for the presence of amide bands. From FT-IR studies, it can ascertain that there is no significant change in the amide regions of collagen. However, due to the presence of pectin there is wide band region due to the interaction. However, the structural integrity of collagen is retained after the interaction of pectin with collagen. Aggregation of molecules is a primary function of collagen, when it is exposed to their nucleation centre. Nucleation point is accelerated, usually occurs when there is a change in the pH which results in order or chaotic aggregation. To evaluate, the aggregation profile, polydispersity index act as a marker. With an increase in the concentration of pectin, the PDI value increased. This again confirms the interaction of pectin with collagen. However, depending on the specific application PDI is preferred or altered. Homogenous or heterogeneous solution can be prepared by altering the concentration of pectin, as an another added advantage of this composite. Fibrillogenesis is one of the special characteristics of fibrillar collagen. Individual monomeric collagen tends to quarter stagger to form microfibrils, sub fibrils and fibrils. Depending on its hierarchical arrangement, the physical strength is increased. During diseased condition, fibrillogenesis is altered, which results in improper functioning of tissues. In the presence of pectin, collagen forms a perfect sigmoid growth pattern with distinct lag (nucleation point), log (fibrils growth) and stationary phase. At a higher concentration of pectin, there is a decrease in lag period, this is probably due to the charged nature of collagen-pectin composite. This property enhances the accelerating growth of collagen fibrils during the wound healing process, specifically diabetic wounds which are usually consume longer time due to its excessive glycosylation in the tissues. Structural conformations can also be ascertained through X-ray diffraction patterns. Collagen has very specific signature peaks 7.44° and 19.78°. Any changes in the 19.78° attributes to the fact that the triple helical nature of collagen is altered. However, in the presence of pectin, collagen-pectin composites exhibits the signatory backbone. This is in accordance with FT-IR results. In addition the nature of collagen in collagen-pectin composite is confirmed through SEM and AFM. From SEM images, it is clearly evident that collagen fibrils are ordered with their D-periodicity. Furthermore, intrinsic gelling nature of pectin ensemble film forming ability of collagen. This characteristic is widely adopted in tissue engineering aspects to prepare collagen films/membranes. As observed in the PDI value of collagen-pectin composites, the aggregation profile is higher at higher concentration, however, the morphological aspects of collagen-pectin is well ordered and maintained. The same aspect is observed through the AFM. Pectin portray an effective defence mechanism against the collagenase enzyme which in turn enhances its structural stability. Pectin tends to interact to both enzyme collagenase and its active sites in collagen. To substantiate the crosslinking profile of collagen-pectin, in silico approach is used in the present study. From computational analysis, it is confirmed that basic amino acids primarily interacts with pectin. Therefore, the physico-chemical interaction profile provides deeper knowledge about collagen-pectin interaction, which is further used as a tissue engineering material for diabetic and anti-aging treatment.

4. Conclusions

The present examination helps to understand the molecular insight of collagen and pectin interactions. The research finding establishes in formulating pectin as a possible diabetic therapeutic agent in preparing collagen scaffold for diabetic wound treatment. Experimental and theoretical works suggest that pectin can act as a compatible component for collagen in formulating scaffold. However, collagen scaffold preparation requires optimization with other cross-linkers and stabilizing agents. Vibrational spectroscopic study of collagen-pectin composites confirms that there is no significant change in the secondary structure conformations in collagen. The aggregation study of collagen in the presence of pectin confirms that pectin induces aggregation due to the charged nature. This resulted in a decrease in the homogeneity of collagen. However, the rate of fibril formation of collagen is not much altered by the presence of pectin and maintains its uniform array. This confirms that in the presence of pectin, collagen tends to aggregate fast in an ordered way. This is the significant finding which helps in the diabetic wound treatment. Fibrillogenesis is one of the critical properties of collagen to attain its functional property. From the XRD study it is observed that there is a minor loss of crystalline nature and acquiring the amorphicity behavior in collagen in the presence of pectin. However, the helicity of the collagen is retained in the presence of polysaccharide. The same has been confirmed by the morphological study. Collagen-pectin complexation finds pectin as the binding pocket on, the protein from the *in silico* analysis. Hence, pectin can be used as one of the therapeutic agent during collagen scaffold preparation for diabetic wound treatments.

Acknowledgement

One of the author (Gladstone Christopher Jayakumar) thanks Council of Scientific & Industrial Research (CSIR), India for awarding Senior Research Fellowship.

Notes

[a] *CSIR-Central Leather Research Institute, Adyar, Chennai-600 020, India. Fax: +91 44 24911589; Tel: +91 44 24911386; E-mail: clrichem@lycos.com*

[b] *Smart Biomaterials Group, Biomaterials Unit, National Institute for Materials Science, 1-1 Namiki, Tsukuba, Ibaraki 305-0044, Japan*

References

1 J. Hong-Fang, L. Xue-Juan, Z. Hong-Yu, *EMBO Reports,*2009, **10**, 194.

2 J.E. Shaw, R.A. Sicree, P.Z. Zimmet, *Diabetes Research and Clinical Practice,*2010, **87,** 4.

3 D. Mohnen, *Current Opinion in Plant Biology,*2008, **11,** 266.

4 W. Zhou, W. Liu, L. Zou, W. Liu, C. Liu, R. Liang, J. Chen, *Colloids and Surfaces B,*2014, **117,** 330

5 R.M. Kay, A.S. Truswell, *American Journal of Clinical Nutrition*,1977, **30,** 171.

6 S. Mokady, *Nutrition&Metabolisn*.,1973,**15,** 290.

7 N.Y. Martínez, I, Cavello, S. Cavalitto, A. Illanes, G. R. Castro. *Colloids Surf. B*.,2014, **117**, 284.

8 T.M.Lin, K.S. Kim, E. Karvinen, A.C. Ivy, *American Journal of Physiology*,1957,**188,** 66.

9 B. Ridley, M.A. O'Neill, D. Mohnen, *Phytochemistry,*2001, **57,**929.

10 Y.S. Chen, C.L. Hsieh, C.C. Tsai, T.H. Chen, W.C. Cheng, C.L. Hu, C.H. Yao, *Biomaterials,*2000, **21,** 1541.

11 D.S. Tuckwell, M.J. Humphries, Crc. Cr. Rev. *Oncol-Hematology,*1993, **15**, 149.

12 J.B. Phillips, S.C.J. Bunting, S.M. Hall, R.A. Brown, *Tissue Engineering,*2005, **11**, 1612.

13 J.E. Scott, *Journal of Anatomy,*1995, **187,**259.

14 N. Usharani, G.C. Jayakumar, S.V. Kanth, J.R. Rao, B. Chandrasekaran, B.U. Nair, *International Journal of Peptide Research and Therapeutics.*2013, **19,** 357.

15 P.T. Sudheesh Kumar, C. Ramya, R. Jayakumar, V.N. Shanti kumar, L. VinothKumar, *Colloids and Surfaces B.*2013, **106,** 109.

16 G.C. Jayakumar, V.K. Swarna, K. Purnasai, B. Chandrasekaran, J.R. Rao, B.U. Nair, *Carbohydrate Polymers*2012, **87,** 1482.

17 N. Usharani, G.C. Jayakumar, V.K. Swarna, J.R. Rao, *Biopolymers*2014, **101**, 903–911.

18 K. Kawakami, *Aaps Pharmscitech*,2010, **11**, 1202.

19 N. Usharani, G.C. Jayakumar, J.R. Rao, B. Chandrasekaran, B.U. Nair, K. Kawakami, *International Journal of Biological Macromolecules*2013, **61**, 404.

20 N. Usharani, G.C. Jayakumar, J.R. Rao, B. Chandrasekaran, B.U. Nair, *Materials Science and Engineering C,*2013, **33**, 4965.

21 G.C. Jayakumar, J.R. Rao, B.U. Nair, *Bioactive Carbohydrates and Dietary Fibres*,2013, **2**,119.

22 J. Torbet, M. Malbouyres, N. Builles, V. Justin, M. Roulet, O. Damour, A. Oldberg, F. Ruggiero, D.J. Hulmes, *Biomaterials*, 2007, **28**, 4268.

23 P.P. Provenzano, R. Vanderby, *Matrix Biology,*2006, **25,** 71.

24 H.L. Heather, S. Shashidhara, M. W. Garrett, *Biopolymers*, 2014, **101,** 329.

A. Grosdidier, V. Zoete, O. Michielin, *Nucleic Acids Research,*2011, **39**, (Web Server issue): W270-7. doi: 10.1093/nar/gkr366.

25 D.W. Ritchie, V. Venkatraman, *Bioinformatics*2010, **26**,2398.

26 G. Macindoe, L. Mavridis, V. Venkatraman, M.D. Devignes, D.W. Ritchie, *Nucleic Acids Research,*2010, **38**,445.

27 N. Usharani, G.C. Jayakumar, V.K. Swarna, J.R. Rao, B. Chandrasekaran, *Journal of Macromolecular Science A Pure and Applied Chemistry,*2012, **49**, 666.

28 A.A.M. Riccardo, B. Joseph, M. Diederick, M. Nicola, D. Marta, G.P. Maurizio, *Carbohydrate Polymers,*2012, **87,** 995.

29 S.G. Arantes, A.D. de Castro, B.S.F. Cury, R.C. Evangelista, *Carbohydrate Polymers*2013, **91,** 135.

30 D.S. Jackson, Recent Advances in Gelatin and Glue Research. 1958, p. 50. Ed. by Stainsby, G. London: Pergamon Press Ltd.

31 J. Gross, *Journal of Experimental Medicine,* 1958, **108,** 215.

32 F.O. Schmitt, J. Gross, J.H. Highberger, *Experimental Cell Research,* 1955,**33**, 326.

33 J. Gross, *Journal of Biophysical and Biochemical Cytology*, 1956, **2,** 261.

34 F.O. Schmitt, *Nature*, 1956, **17**, 503.

35 W.T. Cade, *Physical Theraphy*, 2008, **8,** 1322.

36 F. Zhang, A. Wang, Z. Li, S. He, L. Shao, *Food Science and Nutrition,* 2011, **2**,818.

37 G.J. Cameron, D.E. Cairns, T.J.J. Wess, *Journal of Molecular Biology*, 2007, **372**, 1097.

38 K.E. Kadler, Y. Hojima, D.J. Prockop, *Journal of Biological Chemistry,*1987, **262**, 15696.

39 J.E. Scott, *Journal of Anatomy,*1990, **169**, 23.

40 N. Usharani, G.C. Jayakumar, J.R. Rao, B. Chandrasekaran, B.U. Nair, *Journal of Microscopy,*2014, **253**, 109.

41 G.C. Jayakumar, N. Usharani, K. Kawakami, J. R. Rao, B. U. Nair,*RSC Advances*, 2014 (DOI: 10.1039/C4RA07683D).