Isolation and mass spectrometry based hydroxyproline mapping of type II collagen derived from *Capra hircus* ear cartilage

Priti Prasanna Maity, Debabrata Dutta, Sayan Ganguly, Kausik Kapat, Krishna Dixit, Amit Roy Chowdhury, Ramapati Samanta, Narayan Chandra Das, Pallab Datta, Amit Kumar Das & Santanu Dhara

Collagen II (COLII), the most abundant protein in vertebrates, helps maintain the structural and functional integrity of cartilage. Delivery of COLII from animal sources could improve cartilage regeneration therapies. Here we show that COLII can be purified from the Capra ear cartilage, a commonly available bio-waste product, with a high yield. MALDI-MS/MS analysis evidenced post-translational modifications of the signature triplet, Glycine-Proline-Hydroxyproline (G-P-Hyp), in alpha chain of isolated COLII (COLIIA1). Additionally, thirty-two peptides containing 59 Hyp residues and a few G-X-Y triplets with positional alterations of Hyp in COLIIA1 are also identified. Furthermore, we show that an injectable hydrogel formulation containing the isolated COLII facilitates chondrogenic differentiation towards cartilage regeneration. These findings show that COLII can be isolated from Capra ear cartilage and that positional alteration of Hyp in its structural motif, as detected by newly developed mass spectrometric method, might be an early marker of cartilage disorder.
Collagen is the most abundant protein playing an important role in maintaining the structural and functional integrity of the tissues through their self-interaction. Collagen triple-helix with ∼300 repetitive sequences of Gly-X-Y (where X is often proline and Y is hydroxyproline) is usually identified from nucleotide sequences. Such positional assignment of proline and hydroxyproline (Hyp) ensures stabilization of the triple helix structure through water-bridged intermolecular hydrogen bonding. It is reported that Hyp in collagen is derived from the ascorbic acid-dependent post-translational modification (PTM) of proline residues. This PTM is essential for structural stability of native collagen to minimize pathological dysfunction. COLII, the major protein component in cartilage, consisting of three structural motifs or alpha (a) chains, is enriched with hydroxylation of proline and lysine as well as glycosylation of lysine to maintain its secondary and tertiary structures. Although the primary structure of collagen molecules is usually identified from their nucleotide sequences, there is enormous conflict on their amino acid sequences owing to the changes during PTM, especially hydroxylation of lysine and proline residues. Hyp has an important role in supramolecular fibril network formation of COLII embedded in extracellular matrix. Owing to expression alterations of hydroxylation of proline, lysine, and glycosylation of hydroxylysine during PTM, the resultant COLII may act as auto-antigen epitope in cartilage tissues causing autoimmune response. Accordingly, PTM is an important biochemical fingerprint for detection and verification of collagen functions during modulation of key cellular signaling pathways. Notably, lack of proline hydroxylation in COLIIA1 during PTM promotes dysfunctional collagen assembly in extracellular matrix. Therefore, identification and mapping of Hyp position in signature motif plays an important role to correlate molecular alteration of COLIIA1 with progression of arthritis.

Here we show the isolation of COLII from *Capra* ear cartilage through bio-waste recycling. Post-translationally modified proline residues (i.e., Hyp PTM) could be identified and mapped through tandem mass spectrometry using MASCOT database. Hyp mapping is performed to validate its sequential alteration in Gly–X–Y structural motif in COLIIA1. This identification may contribute to the prediction of dysfunctional collagen leading to arthritis as well as molecular identification of COLII from other sources. Moreover, the isolated COLII is transformed into hydrogel along with Pluronic F127 in combination with *Capra* adipose tissue-derived stem cells (ADMSCs) towards differentiation of chondrogenic lineage in vitro.

**Results**

**Isolation and identification of COLII from *Capra* ear cartilage.** Isolation of COLII was optimized using 0.1% (w/v) pepsin digestion followed by 1.2 M NaCl precipitation to produce yield of ∼55% (on dry weight basis) (Supplementary Fig. 1). Hyp content was estimated to be ∼118 mg/g of COLII. SDS-PAGE profile of (Supplementary Fig. 1a) isolated COLII showed two prominent bands at 122 kDa and 200 kDa for a chain and β sheet, respectively. Purity of COLII was found to be higher in case of 1.2 M NaCl precipitation as compared to that of 0.9 M. Moreover, isolated COLII was identified using anti-COLII antibody (Abcam, USA) (Fig. 1b) and absence of elastin in COLII was confirmed by anti-elastin antibody (Abcam, USA) using western blot analysis (Supplementary Fig. 2).

**Physico-chemical characterization of COLII Fourier Transforms Infrared (FTIR) spectroscopy.** FTIR spectrum represents major absorption bands observed at 3336 cm⁻¹, 1658 cm⁻¹, 1555 cm⁻¹, and 1240 cm⁻¹ were attributed to amide A, amide I, amide II and amide III, respectively, for purified COLII (Fig. 2a). The spectrum of Amide I band, after deconvolution (Fig. 2b), reveals 1624 cm⁻¹, 1638 cm⁻¹, 1659 cm⁻¹, 1684 cm⁻¹ for β-sheets, random coils, α-helices and β-sheets, respectively. Similarly deconvolution of Amide III (Fig. 2c) reveals peaks at 1200–1350 cm⁻¹ with 1339 cm⁻¹, 1315 cm⁻¹, 1240 cm⁻¹ representing CH₂ deformation, –C(=O)H wagging and CN stretching (also NH deformation), respectively.

**Circular dichroism (CD) spectra of COLII.** CD spectra of COLII in a range of 25–45 °C is shown in Fig. 3a. The spectra show a dichroic signal maximum at 221 nm (positive band), minimum at 198 nm (negative band) with consistent cross over point (zero rotation) at about 212 nm ensuring characteristic triple helical conformation of COLII. Figure 3b represents corresponding mean molar ellipticity, [θ]221, as a function of temperature. Values of [θ]221 decreased with the increase of temperature due to denaturation of collagen triple helical structure. Denaturation temperature (T_d) was measured to be 43 °C. The percentage of intactness and denaturation of COLII at different temperatures have been shown in Supplementary Table 1.

**Field emission scanning electron microscopy (FESEM) of COLII.** FESEM images depicted the overlapping COLII fibers with characteristic D-banding pattern (Fig. 4a, b). In collagen fibers, tropo-collagens were aligned in a parallel manner while maintaining the gap overlapping pattern. Results showed alternate light/dark-band pattern, referring to D spacing of collagen associated with regular arrangement of the molecules. The calculated D-spacing value was ∼67 nm, similar to the values reported elsewhere.

**Amino acid profiling of COLII.** Amino acid profiling of COLII was carried out by HPLC analysis and the results showed higher contents of G, P, and Hyp residues (i.e., 302, 99, and 118 residues per 1000 amino acids residues, respectively) compared to the contents of tyrosine, cysteine, histidine and methionine residues.
(i.e., 5, 20, 5, and 10 residues, respectively) (Supplementary Table 2). Similar results for cartilage COLII were reported elsewhere.

Proteomic characterization of COLII. Peptide extraction and MALDI MS analysis: MALDI-TOF/TOF analysis of COLII A1 band (~122 kDa) from SDS-PAGE exhibited peptide fragments of COLII A1 after tryptic digestion (Fig. 5; Supplementary Table 3). Individual peptides were further used for MS/MS analysis by adopting reported protocol published elsewhere.

Peptide mass fingerprinting (PMF) of COLII A1 for PTM analysis: The full length primary sequence of COLII A1 of Capra hircus was unavailable in MASCOT database. Therefore, our evaluation of COLII A1 sequence was based on amino acid sequence of Bos taurus (Uni-Prot accession number: P02459) and Homo sapiens (Uni-Prot accession number: P02458), which are closely related to Capra hircus. Primary amino acid sequence of

**Fig. 2** FTIR analysis of COLII. a FTIR spectrum of COLII; deconvoluted spectra of b Amide I and c Amide III showing characteristic signature of COLII

**Fig. 3** CD analysis of purified COLII. a CD spectra of COLII obtained at different temperatures (25-45 °C) and b at 221 nm showing denaturation temperature at 43 °C
Capra hircus COLIIA1 (KEGG entry: 100860743) derived from genome sequence showed 98% identity with Bos taurus COLIIA1 (Supplementary Fig. 3). The obtained peptide fragments ranging between 700 and 3500 Dalton from in-gel digestion showed ~30% coverage in full length sequence of Bos taurus COLIIA1 (Supplementary Fig. 4). To investigate variable modification of proline oxidation, SwissProt database searching option was adopted for detection of Hyp PTM in fragments peptides of COLIIA1 through addition of 16 Da to proline residues depicting Hyp. MASCOT identified 32 peptides (Table 1) where 59 Hyp PTM were present in COLIIA1.

Site specific characterization of Hyp PTM in G-X-Y triplets: MALDI tandem mass spectrometry (MS-MS) identified fragmented ions derived from tryptic peptides of COLIIA1 for site specific characterization of Hyp PTM. Therefore, 1500 and 1814 Da peptides were selected for MS/MS fragmentation shown in Fig. 6a, c. Fragment ion analysis of 1500 and 1814 Da peptides identified Hyp position at 1018, 1019 number amino acid of G-Hyp-Hyp, 893 of G-P-Hyp and 899, 1034 of G-X-Hyp, the G-X-Y triplets present in COLIIA1 primary sequence (Fig. 6b, d; Table 1; Supplementary Fig. 4). Hyp positions were annotated by fragmented ions (b and y ions) of 1500 and 1814 Da peptides (Fig. 6b, d).

Assessment of chondrogenic potential of COLII derived injectable hydrogel. Preparation of thermo-reversible hydrogel (CP): Thermo-responsive hydrogel containing COLII and Pluronic F127 copolymer was prepared at 4 °C. The gelation was evidenced within 30 s after reaching the temperature at ~32 °C. Gelation was visualized by vial-inversion method as shown in Fig. 7a. The temperature sweep experiment evidenced sharp change in storage modulus (G) as shown in Fig. 7b. The 10² fold increase in G value supported the increase in storage modulus thereby gel formation. At below-ambient temperature, the hydrogel was mobile enough and showed minimal storage modulus in the sub-Pascal range confirming the Newtonian fluidity. After increasing temperature, the system responded.

![Fig. 4 FESEM images of COL II. a Showing the overlapping fibrilar structure and b characteristic D-spacing value of ~67 nm](image)

![Fig. 5 Mass spectrometric analysis of purified COLII. MALDI-MS spectrum of tryptic peptides derived from COLIIA1 protein band and identification with MASCOT histogram showing significant (p < 0.05) MASCOT score (82) for COLIIA1 of Bos taurus](image)
Table 1 COLIIA1 peptides of Capra hircus containing Hyp residues identified by peptide mass fingerprinting analysis

| m/z       | Range | Amino acid sequences         | No. of P residue(s) | No. of Hyp residue(s) |
|-----------|-------|------------------------------|--------------------|-----------------------|
| 852.4903  | 267-275 | GPPGPQGAR                    | 3                  | 1                     |
| 1976.0676 | 267-287 | GPPGPQGARGFGPGLPGVK          | 6                  | 2                     |
| 1584.8541 | 420-437 | GSAGAPIAGAPFGPGR             | 4                  | 3                     |
| 1353.6891 | 495-509 | GEPGAGPAPGPGERGAPGNGR        | 5                  | 4                     |
| 1921.9713 | 495-515 | GEPGAGPAPGPGERGAPGNGR        | 5                  | 4                     |
| 1366.6988 | 528-542 | GAPGERPGSLAPG               | 3                  | 1                     |
| 1706.8428 | 543-560 | GANQDGPGRGPGGLPGAR           | 6                  | 2                     |
| 1326.7555 | 561-574 | GLTGRPGAGPQGR               | 2                  | 1                     |
| 2045.0664 | 575-596 | VGPSAGPEGEDGRPQPQGAR         | 6                  | 3                     |
| 853.4861  | 621-629 | GLPGAPGLR                   | 2                  | 1                     |
| 869.4979  | 621-629 | GLPGAPGLR                   | 3                  | 2                     |
| 1326.7555 | 621-634 | GLPGAPGLRGGLPGK             | 4                  | 3                     |
| 1353.6891 | 630-653 | GLTGRPGAGPQGR               | 3                  | 1                     |
| 1366.6988 | 630-653 | GLTGRPGAGPQGR               | 5                  | 2                     |
| 1679.8304 | 655-653 | DGETGAAAGPQPAGPAGER         | 4                  | 1                     |
| 878.3628  | 681-720 | GIIQGPGLQGK                 | 1                  | 1                     |
| 1128.5859 | 720-731 | GIIQGPGLQGK                 | 2                  | 1                     |
| 2913.5118 | 732-764 | GAAGPAGPGQGPPQGLQMPGERGAAGIAGPK | 7                | 4                     |
| 2106.0665 | 825-848 | GETGPPPGAFAGPGDGQPGAK       | 6                  | 1                     |
| 1500.7894 | 888-904 | GAQGPAPATGPGAAPGR           | 3                  | 2                     |
| 912.5034  | 981-989 | GIVGLPGQKR                  | 1                  | 1                     |
| 1328.7270 | 993-1006 | GFGLPGSSPGEPGK             | 4                  | 2                     |
| 2225.1735 | 993-1016 | GFGLPGSSPGEPGK             | 5                  | 2                     |
| 1798.9796 | 1071-1036 | GPPGPVPPGLTPGAEQGR        | 7                  | 2                     |
| 1814.9789 | 1071-1036 | GPPGPVPPGLTPGAEQGR        | 7                  | 3                     |
| 2513.2866 | 1066-1084 | GDRGETGAVPAPGGPQGPGAPGPIGK | 7                | 2                     |
| 1567.8021 | 1089-1106 | GEAGAQPGMPGAPFAGAR       | 3                  | 1                     |
| 928.7476  | 1107-1115 | GMPPGPQGPR                | 3                  | 2                     |
| 2703.3415 | 1134-1163 | GFTGLOQLPGPQGSPDGASPAGPSGPR | 7               | 2                     |
| 2721.3574 | 1134-1163 | GFTGLOQLPGPQGSPDGASPAGPSGPR | 7               | 3                     |
| 1519.8262 | 1175-1190 | DGANGIPPIGPPGPR            | 5                  | 3                     |
| 1551.7982 | 1175-1190 | DGANGIPPIGPPGPR            | 5                  | 5                     |

Fig. 6 Hyp-PTM mapping of COLIIA1 peptides. MALDI MS/MS spectra and fragment ion annotations of a, b 1500 Da and c, d 1814 Da peptides showing specific position of Hyp PTM in the sequences. "Pox" represents the Hyp residues.
demands of COLII, various methods are being explored by cutting edge alternative therapeutic approach. To meet increasing demand or using injectable gel based delivery vehicle could be a potential solution.

Cartilage regeneration using COLII by oral nutrient supplementation or using injectable gel based delivery vehicle could be a cutting edge alternative therapeutic approach. To meet increasing demands of COLII, various methods are being explored by researchers to isolate this protein from different sources. Capra ear cartilage is one such bio-waste resources, which is not remarkably explored for isolation of COLII.

Here we show a simple, rapid isolation process of COLII in a cost effective way. The process demonstrates a simple route to separate skin from Capra ear as an alternative towards easy isolation of cartilage using hypertonic solution following 0.04% pepsin digestion at 37 °C for 72 h, a novel addition to the existing process. Pepsin (0.1%) based tissue digestion is performed at room temperature to increase the activity of pepsin resulting in considerable higher yield (≥55%)23. This enzymatic digestion also enabled removal of N-terminal and C-terminal of non-helical COLII chain (telopeptide region) through breakdown of intermolecular crosslinks usually formed through aldol condensation10,11. Removal of telopeptide (N-terminal and C-terminal) and breakdown of secondary structures are necessary to reduce antigenicity, while increasing biocompatibility and bio-degradability11. The existing process for isolating COLII from other resources reported maximum yield of 9–55% (on dry weight basis) using 1% pepsin digestion followed by precipitation.

Isolation and identification of ADMSCs from Capra hircus: The isolated ADMSCs were identified by immune-fluorescence (IF) staining and real time PCR (RT-PCR). After 3rd passage, isolated cells from Capra adipose tissue showed distinct expression of surface marker, CD 44 along with the absence of CD 31 depicting ADMSCs characteristics. Positive expression of surface marker, CD 44 along with the absence of CD 31 was confirmed by RT-PCR analysis as well (Supplementary Fig. 5). Trilineage (adipogenic, osteogenic and chondrogenic) differentiation potential of isolated ADMSCs were also assessed after 21 days of differentiation studies. After adipogenic differentiation, accumulation of lipid droplets in cell cytoplasm was observed through Oil Red O staining, whereas migration of cells in closer contact forming occasional clumps as well as positive in Alcian blue staining were witnessed after chondrogenic differentiation. When supplemented with the osteogenic medium, cells deposited extracellular calcium crystals, which were detected through Alizarin Red S staining. However, such trilineage differentiation characteristics were absent in control supplemented with regular medium (Supplementary Fig. 6)22.

Assessment of cytotoxicity and cell proliferation of CP hydrogel: Adhesion, viability and morphological characteristics of ADMSCs seeded on CP hydrogel were analysed by live-dead and Rhodamine-DAPI staining. Figure 8 shows relatively higher cell adhesion and proliferation on 5d as compared to 3d culture indicating cytocompatible nature of the hydrogel. Presence of insignificant dead cells after live-dead staining also signifies its non-cytotoxic nature. After 3d and 5d culture, Rhodamine-DAPI staining revealed similar morphology of ADMSCs within CP hydrogel as compared to TCP.

Gene expression analysis and sulfated glycosaminoglycan (sGAG) quantification: Chondrogenic potential of CP hydrogel was evaluated by analysing cartilage related gene (COLII) expression using RT-PCR. Expression of COLII was upregulated for cells grown within CP hydrogel as compared to that of F127 hydrogel (P ≤ 0.0001) (Fig. 9a, b). Further, accumulation of sGAG was significantly higher with CP hydrogel (13.63 ± 1.35 mg) in comparison with control (5.64 ± 0.62 mg) (P < 0.0008) (Fig. 9c).

**Discussion**

Cartilage regeneration using COLII by oral nutrient supplementation or using injectable gel based delivery vehicle could be a cutting edge alternative therapeutic approach. To meet increasing demands of COLII, various methods are being explored by

Researchers to isolate this protein from different sources. Capra ear cartilage is one such bio-waste resources, which is not remarkably explored for isolation of COLII.

Here we show a simple, rapid isolation process of COLII in a cost effective way. The process demonstrates a simple route to separate skin from Capra ear as an alternative towards easy isolation of cartilage using hypertonic solution following 0.04% pepsin digestion at 37 °C for 72 h, a novel addition to the existing process. Pepsin (0.1%) based tissue digestion is performed at room temperature to increase the activity of pepsin resulting in considerable higher yield (≥55%)23. This enzymatic digestion also enabled removal of N-terminal and C-terminal of non-helical COLII chain (telopeptide region) through breakdown of intermolecular crosslinks usually formed through aldol condensation10,11. Removal of telopeptide (N-terminal and C-terminal) and breakdown of secondary structures are necessary to reduce antigenicity, while increasing biocompatibility and bio-degradability11. The existing process for isolating COLII from other resources reported maximum yield of 9–55% (on dry weight basis) using 1% pepsin digestion followed by precipitation.
with 0.9 M NaCl in 5–7 days. The present study demonstrates ~55% yield of COLII from Capra ear cartilage using 1/10th dose of pepsin followed by precipitation with 1.2 M NaCl in 48 h, notably reducing the processing cost16,18,24. FTIR study revealed changes in the secondary structure of isolated COLII. The shift of amide A spectral region to marginally lower value (3336 cm⁻¹) as compared to that of the other proteins (3400–3440 cm⁻¹) indicates formation of H-bonds between N–H groups and other groups, resulting in a stable triple helix structure25. The intense peak of amide I (1658 cm⁻¹) associated with amide residues indicates that intermolecular crosslinks and ß turns in isolated COLII remained unchanged, similar to that of native cartilage26. On the other hand, random coil state was shifted towards lower wave number indicating slight denaturation during isolation process at room temperature13. Presence of amide II band (1553 cm⁻¹) indicates retention of the triple helical structure of the isolated protein26. Amide III bands at 1200–1350 cm⁻¹ is known to be the fingerprint region of collagen molecule. Changes in this region is directly related to changes in the native tripleptide (Gly-Pro-Hyp)₃ sequences of collagen and endorsed to different identification of a same molecule27. The deconvoluted peaks in this region strongly suggest that the signature sequence of COLII (Gly-Pro-Hyp) remained unaffected in the isolated protein, which was in agreement to the result reported by Bachmann et al.27. This finding includes deconvolution of specific band recommends that the extracted protein of Capra ear cartilage is COLII and retained native intermolecular crosslinking during isolation and purification process.

CD is an essential tool for rapid evaluation of secondary structure and protein folding through unequal absorption of left-handed and right-handed circularly polarized light while passing through prism/filter with the sinusoidal oscillation of electric field in a single plane. Herein, the CD was effectively utilized to study the extent of denaturation of the COLII, which was isolated by a temperature dependent process. In proteins, chromophores in polypeptide backbone are aligned in arrays and their optical properties were explored by FESEM. The isolated COLII via acetic acid/pepsin based digestion process, exhibited fibrillar and multi-layered structure similar to native structures as reported elsewhere16,18. The tropo-collagens in collagen fibers are aligned in a parallel manner while maintaining the repeating gap overlapping pattern. This gap overlapping pattern is referred to D-spacing (~67 nm length) associated with regular arrangement of collagen microfibrils17.

Collagen molecule consists of a unique triple-helical structure with three left-handed polyproline II like chains supercoiled in a right-hand manner along a common axis. To confirm this structure, the glycine residues at every third position should be present in the amino acid sequence. Therefore, amino acid profiling of COLII indicates presence of high content of glycine, hydroxyproline, and proline with small amounts of tyrosine, histidine and methionine per 1000 amino acids residues. The high glycine content in α-chain of COLII signifies its presence in every third residue in the sequence, except in first 14 amino acid residues of the N-terminus and first 10 amino acid residues of the C-terminus18. Presence of high content of other amino acids (proline and hydroxyproline) in α-chain contributed towards formation of planar peptide bonds. These assumptions indicates to the construction of correct model of Capra ear cartilage derived COLII as a (Gly-X-Y)₃ pattern. Indeed, it is the most commonly reported triplet in collagen α chain space16.

Peptide mapping of COLII obtained through precipitation in different NaCl concentration exhibited similar migration bands, which were composed of three identical α-chains[α(II)₃] with molecular weight 122 kDa reported elsewhere18. Bands obtained in SDS-PAGE and subsequent western blot analysis using anti-collagen II antibody (Abcam, USA) indicated high purity of isolated COL II18,16,29. Furthermore, western blot analysis using anti-elastin antibody (Abcam, USA) confirmed absence of any elastin protein in the isolated COLII from Capra ear cartilage. The result indicates that NaOH pre-treatment for Capra ear cartilage is necessary for the removal of non-collagenous protein from tissue30.

The predicted and partial genome sequences of Capra hircus and its collagen gene isoforms are available in gene database. Till date, Capra hircus COLIIA1 protein sequence is partially available in the SwissProt; but its primary sequence is available in the
Isolated COLII was blended with Pluronic F127 for preparation of COLII-Pluronic (CP) hydrogel. Pluronic F127 is non-toxic FDA approved poly (ethylene oxide)/poly(propylene oxide)/poly (ethylene oxide) (PEO-PPO-PEO) triblock copolymers. Owing to non-toxicity and thermal gelation property, this polymer is considered as an excellent drug and cell delivery vehicle. Aqueous solution of this polymer undergoes sol-to-gel transition above a certain lower critical gelation temperature owing to amphiphilic solution of this polymer undergoes sol-to-gel transition above a certain lower critical gelation temperature owing to amphiphilic polymer aggregation and micelle formation. At mole-
Dry weight collagen x 100

(Wet weight of sample – Moisture content of sample)

\[
\% \text{Yield (dry weight basis)} = \frac{\text{Dry weight collagen}}{\text{Total collagen prepared}} \times 100
\]

**SDS-PAGE and western blot analysis.** SDS-PAGE was performed using 4% (w/v) stacking gel and 8% (w/v) separating gel. The purified protein was mixed with 6X loading dye at 1:3 (w/v) ratio and subsequently loaded in stacking gel. Protein marker (MW range 25–250 kDa; BioRad, India) was run in a separate well along with samples. Electrophoresis was performed using mini dual vertical electrophoresis unit at 90 V for 2 h (BioRad, India). The obtained band was observed by Coomassie blue staining. After excision of proteins through SDS-PAGE, the gel was electro-blotted onto a nitrocellulose membrane (Millipore, USA) in tris-glycine buffer at 90 V for 2 h. Membranes were incubated with anti-collagen II antibody and anti-elastic antibody (1:5000) (Abcam, USA) at 4°C overnight after blocking with 3% BSA in PBS. The blots were washed with PBS (0.05% Tween 20 in PBS) and incubated with horseradish peroxidase (HRP) conjugated secondary antibody (1:6000) for 2 h at RT. BioRad ECL western blotting substrate was used to visualize the immune-reactive proteins as per the manufacturer's instructions (Thermo Scientific, USA). Images were observed by chemi-luminescence.

Fourier transform infrared spectroscopy (FTIR): FTIR spectrum of purified protein was collected through KBr pelletation technique in the range of 4000–700 cm\(^{-1}\) using Thermo Nicolet Spectrophotometer (Model NEXUS-870; Thermo Nicolet Corporation, Madison, WI).

Circular dichroism (CD): CD spectra were collected to analyze the secondary structure of acid/pepsin-solubilized isolated proteins fraction under different digestion conditions. CD profile (1 mg) was dissolved in 1:10 (w/v) acetic acid (0.05 M) solution and placed into a quartz cell with a path length of 1 mm. CD spectra of protein solution were recorded from 280 to 190 nm with a step size of 1.0 nm and bandwidth 1.0 nm at scan speed 100 nm/min after subtraction of solvent spectrum. Scanning electron microscopy (SEM): FESEM (EVO 60, Carl Zeiss, Germany) of the isolated and purified protein was carried out after gold coating using plasma coater for the 30 s under high vacuum.

Amino acid profiling: For amino acid analysis, purified protein was hydrolyzed with 6 N HCl for 24 h at 120 °C. The resultant mixture was analysed by an Agilent 1200 HPLC system (Agilent, USA) with a 6 N HCl for 24 h at 120 °C. The resultant mixture was analysed by an Agilent 1200 HPLC system (Agilent, USA) with a 6 N HCl for 24 h at 120 °C. The resultant mixture was analysed by an Agilent 1200 HPLC system (Agilent, USA) with a 6 N HCl for 24 h at 120 °C.

Isolation of COLII from Capra hircus. Neophydrone was mixed with 6X loading dye at 1:3 (w/v) ratio and subsequently loaded in stacking gel. Protein marker (MW range 25–250 kDa; BioRad, India) was run in a separate well along with samples. Electrophoresis was performed using mini dual vertical electrophoresis unit at 90 V for 2 h (BioRad, India). The obtained band was observed by Coomassie blue staining. After excision of proteins through SDS-PAGE, the gel was electro-blotted onto a nitrocellulose membrane (Millipore, USA) in tris-glycine buffer at 90 V for 2 h. Membranes were incubated with anti-collagen II antibody and anti-elastic antibody (1:5000) (Abcam, USA) at 4°C overnight after blocking with 3% BSA in PBS. The blots were washed with PBS (0.05% Tween 20 in PBS) and incubated with horseradish peroxidase (HRP) conjugated secondary antibody (1:6000) for 2 h at RT. BioRad ECL western blotting substrate was used to visualize the immune-reactive proteins as per the manufacturer's instructions (Thermo Scientific, USA). Images were observed by chemi-luminescence.

Fourier transform infrared spectroscopy (FTIR): FTIR spectrum of purified protein was collected through KBr pelletation technique in the range of 4000–700 cm\(^{-1}\) using Thermo Nicolet Spectrophotometer (Model NEXUS-870; Thermo Nicolet Corporation, Madison, WI).

Circular dichroism (CD): CD spectra were collected to analyze the secondary structure of acid/pepsin-solubilized isolated proteins fraction under different digestion conditions. CD profile (1 mg) was dissolved in 1:10 (w/v) acetic acid (0.05 M) solution and placed into a quartz cell with a path length of 1 mm. CD spectra of protein solution were recorded from 280 to 190 nm with a step size of 1.0 nm and bandwidth 1.0 nm at scan speed 100 nm/min after subtraction of solvent spectrum. Scanning electron microscopy (SEM): FESEM (EVO 60, Carl Zeiss, Germany) of the isolated and purified protein was carried out after gold coating using plasma coater for the 30 s under high vacuum.

Amino acid profiling: For amino acid analysis, purified protein was hydrolyzed with 6 N HCl for 24 h at 120 °C. The resultant mixture was analysed by an Agilent 1200 HPLC system (Agilent, USA) with a fluorescent detector (FLD) after derivative formation with 2% ninhydrin (MP Biomedicals, USA). For protein analysis, hydroxyproline identification, 9-fluorenylmethylcarbonyl (FMOC-CI) (SRL, India) was applied to derivatise the sample. The derivatized sample was loaded into a HPLC system (Agilent, USA) with a fluorescence detector (FLD) after derivative formation with 2% ninhydrin (MP Biomedicals, USA). For protein analysis, hydroxyproline identification, 9-fluorenylmethylcarbonyl (FMOC-CI) (SRL, India) was applied to derivatise the sample. The derivatized sample was loaded into a HPLC system (Agilent, USA) with a fluorescence detector (FLD) after derivative formation with 2% ninhydrin (MP Biomedicals, USA). For protein analysis, hydroxyproline identification, 9-fluorenylmethylcarbonyl (FMOC-CI) (SRL, India) was applied to derivatise the sample. The derivatized sample was loaded into a HPLC system (Agilent, USA) with a fluorescence detector (FLD) after derivative formation with 2% ninhydrin (MP Biomedicals, USA). For protein analysis, hydroxyproline identification, 9-fluorenylmethylcarbonyl (FMOC-CI) (SRL, India) was applied to derivatise the sample. The derivatized sample was loaded into a HPLC system (Agilent, USA) with a fluorescence detector (FLD) after derivative formation with 2% ninhydrin (MP Biomedicals, USA).

**Assessment of trilignear differential potential of ADMSCs:** For adipogenic differentiation, ADMSCs were plated at a density of 1 x 10^3 in each well of 6-well plate. After adhesion of cells, the DMEM media was replaced with lineage-specific differentiation medium. For adipogenic differentiation media contained: DMEM (high glucose), 10 ml FBS, 0.0393 mg dexamethasone (Himedia, 11.1 mg 3- isobuty1-1-methylxanthine (Sigma), 7.16 mg indomethacin (Sigma) and 5.73 mg isobocastadione (Sigma). Chondrogenic differentiation medium contained: DMEM (high glucose), 2–5% FBS (Gibco, USA), 0.00393 mg dexamethasone (Himedia, India), 5.79 mg ascorbic acid-2-phosphate (Sigma, USA), 1 ml ITS-X (Life Technologies), 0.402 mg i-proline and 10 μl TGF-β3 (Gibco, USA). Osteogenic differentiation medium contained: DMEM (high glucose), 10% FBS, 0.0393 mg dexamethasone (Himedia, India), 216 mg β-glycerophosphate (Sigma, USA) and 5 mg ascorbic acid (Sigma, USA).

Media was replaced in every 3 days up to 21 days of differentiation study. At the end of the experiment, cells were rinsed with PBS and fixed with 4% paraformaldehyde for 60 min. Cells were washed with distilled water (D/W). After paraformaldehyde fixation, cells were rinsed with 60% isopropanol followed by incubation with Oil Red O solution for 5 min. The cells were washed with D/W followed by counterstaining with Hematoxylin for 5 min. The fixed cells were dehydrated through an ethanol series with 2% Alizarin Red S solution at room temperature for 10 min followed by washing with D/W. After staining, cells were visualized under the light microscope (AxioVision, Zeiss, Germany).
In vitro cytocompatibility study: CP hydrogel was formed within the wells of a 24-well cell culture plate surface (1 ml/well) for in situ gelation at 37 °C for 5 min. The harvested ADMSCs from passage three were seeded in equal numbers (50,000 cells/well) on CP hydrogel as well as tissue culture plate (TCP) as a control. The live-dead assay was performed by using Live/Dead staining kit (Invitrogen, USA). The cell-hydrogel construct and TCP was cultured at 37 °C, 5% CO2 incubator and counted in a haemocytometer. Cells were again centrifuged and cell pellets were re-suspended in fresh medium and counted in a haemocytometer. The experiments were carried out in triplicate.

3. Ying Chow, W. et al. Hydroxyproline ring pucker causes frustration of helix parameters in the collagen triple helix. Sci. Rep. 5, 12556 (2015).

4. Weis, M. A. et al. Location of 3-hydroxyproline residues in collagen types I, II, III, and V/XI implies a role in fibril supramolecular assembly. J. Biol. Chem. 285, 2580–2590 (2010).

5. Karsdal, M. A. et al. Extracellular matrix remodeling: the common denominator in connective tissue diseases. Assay Drug Dev. Technol. 11, 70–92 (2013).

6. Ottani, V., Martini, D., Franchi, M., Ruggeri, A. & Raspanti, M. Hierarchical structures in fibrillar collagens. Micron 33, 587–596 (2002).

7. Duan, G. & Walther, D. The roles of post-translational modifications in the context of protein interaction networks. PLoS Comput. Biol. 11, 1–23 (2015).

8. Basak, T. et al. Comprehensive characterization of glycosylation and hydroxylation of basement membrane collagen IV by high-resolution mass spectrometry. J. Proteome Res. 15, 245–258 (2016).

9. Tenny, R., Valli, M., Rossi, A. & Cetta, G. Possible role of overglycosylation in the type I collagen triple helical domain in the molecular pathogenesis of osteogenesis imperfecta. Am. J. Med. Genet. 45, 252–256 (1993).

10. Dominguez, L. J., Barbagallo, M. & Moro, L. Collagen overglycosylation: a biochemical feature that may contribute to bone quality. Biochem. Biophys. Res. Commun. 330, 1–4 (2005).

11. Millares, P. et al. Proteomic profiling and protein identification by MALDI-TOF mass spectrometry in unsequenced parasitic nematodes. PLoS ONE 7, 11 (2012).

12. Reddy, G. K. & Enwemeka, C. S. A simplified method for the analysis of bone and soft tissue biologicals and hydroxyproline. Clin. Biochem. 32, 3–11 (1999).

13. Holmes, R., Kirk, S., Tronci, G., Yang, X. & Wood, D. Influence of telopeptides on the structural and physical properties of polymeric and monomeric acid-soluble type I collagen. Mater. Sci. Eng. C 77, 823–827 (2017).

14. De Campos Vidal, B. & Mello, M. L. S. Collagen type I amide I band infrared spectroscopy. Micron 42, 283–289 (2011).

15. Chadea, F., Hø, A., Le, Bellot-gurlet, L. & Ina, R. Curve-fitting micro-ATR-FTIR studies of the amide I and II bands of type I collagen in archaeological bone materials. e-Ps 6, 129–137 (2009).

16. Belbachir, K., Noeren, R., Gouspillou, G. & Petibois, C. Collagen types analysis and differentiation by FTIR spectroscopy. Anal. Bioanal. Chem. 395, 829–837 (2009).

17. Louis-Jeune, C., Andrade-Navarro, M. A. & Perez-Iratxeta, C. Prediction of protein secondary structure from circular dichroism using theoretically derived spectra. Proteins Struct. Funct. Bioinform. 80, 374–381 (2012).

18. Cao, H. & Xu, S.-Y. Purification and characterization of type II collagen from chick chondreal cartilage. Food Chem. 108, 439–445 (2008).

19. Pal, P. et al. Accelerating full thickness wound healing using collagen sponge of mirgal fish (Cirrhinus cirrhosus) scale origin. Int. J. Biol. Macromol. 93, 1507–1518 (2016).

20. Jeewithan, E. et al. Type II collagen and gelatin from silvertip shark (Carcharhinus albimarginatus) cartilage: isolation, purification, physicochemical and antioxidant properties. Mar. Drugs 12, 3852–3873 (2014).

21. Mazumder, S. K., Das, S. K., Rahim, S. M. & Ghaffar, M. A. Temperature and diet effect on the pepsin enzyme activities, digestive somatic index and relative gut length of Malabar blood snapper (Lutjanus malabaricus Bloch & Schneider, 1801). Aquac. Res. 49, 1–7 (2018).

22. Mohammad-fauzi, N., Ross, P. J., Maga, E. A. & Murray, J. D. Impact of source tissue and ex vivo expansion on the characterization of goat mesenchymal stem cells. J. Anim. Sci. Biotechnol. 6, 22 (2015).

23. Ganguly, S. et al. Green reduced graphene oxide toughened semi-IPN monolith hydrogel as dual responsive drug release system: rheological, physicochemical, and electrical evaluations. J. Phys. Chem. B 122, 7201–7218 (2018).

24. Meyonga, J. H., Cole, C. G. B. & Duodu, K. G. Fourier transform infrared (FTIR) spectroscopic study of acid soluble collagen and gelatin from skins and bones of young and adult Nile perch (Lates niloticus). Food Chem. 86, 325–332 (2004).

25. Hanifi, A., McCarthy, H., Roberts, S. & Pleshko, N. Fourier transform infrared imaging and infrared fiber optic probe spectroscopy identify collagen type in connective tissues. PLoS ONE 8, 11–13 (2013).

26. Bachmann, L., Gomes, A. S. L. & Zezell, D. M. Collagen absorption bands in heated and rehydrated dentine. Spectrochim. Acta Part A 62, 1045–1049 (2005).

27. Muyonga, J. H., Cole, C. G. B. & Duodu, K. G. Fourier transform infrared (FTIR) spectroscopic study of acid soluble collagen and gelatin from skins and bones of young and adult Nile perch (Lates niloticus). Food Chem. 86, 325–332 (2004).

28. Hanifi, A., McCarthy, H., Roberts, S. & Pleshko, N. Fourier transform infrared imaging and infrared fiber optic probe spectroscopy identify collagen type in connective tissues. PLoS ONE 8, 11–13 (2013).

29. Bachmann, L., Gomes, A. S. L. & Zezell, D. M. Collagen absorption bands in heated and rehydrated dentine. Spectrochim. Acta Part A 62, 1045–1049 (2005).
28. Lopes, J. L. S., Miles, A. J., Whitmore, L. & Wallace, B. A. Distinct circular dichroism spectroscopic signatures of polypropylene II and unordered secondary structures: applications in secondary structure analyses. *Protein Sci.* **23**, 1765–1772 (2014).

29. Kambic, H. E. & Mcdevitt, C. A. Spatial organization of types I and I1 collagen in the canine meniscus. *J. Orthop. Res.* **23**, 142–149 (2005).

30. See, S. F., Ghassem, M., Mamot, S. & Babji, A. S. Effect of different pretreatment (IFAS) on functional properties of African catfish (Clarias gariepinus) skin gelatin. *J. Food Sci. Technol.* **52**, 753–762 (2015).

31. Ganguly, S. et al. Polysaccharide and poly(methacrylic acid) based biodegradable elastomeric biocompatible semi-IPN hydrogel for controlled drug delivery. *Mater. Sci. Eng. C* **92**, 34–51 (2018).

32. Ganguly, S. et al. Design of psyllium-g-poly(acrylic acid-co-sodium acrylate)/cloisite 10A semi-IPN nanocomposite hydrogel and its mechanical, rheological and controlled drug release behaviour. *Int. J. Biol. Macromol.* **111**, 983–998 (2018).

33. Ganguly, S. et al. Mechanically robust dual responsive water dispersible-graphene based conductive elastomeric hydrogel for tunable pulsatile drug release. *Ultrason. Sonochem.* **42**, 212–227 (2018).

34. Fu, C., Ren, F., Zhang, Q., Luo, G. & Zhang, L.-M. Effects of collagen incorporation on thermogelation and hydrogel characteristics of aqueous Pluronic F127 copolymer system. *Colloid Polym. Sci.* **293**, 2191–2200 (2015).

35. Raghavan S. R. & Capriano, B. H. Molecular Gels: Materials with Self-Assembled Fibrillar Networks (eds. Terech, P. & Weiss, R. G.) Ch. 8, 233–244 (Springer, Dordrecht, 2006). https://www.springer.com/in/book/9781402033520.

36. Bourin, P. et al. Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: a joint statement of the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT). *Cytotherapy* **15**, 641–648 (2013).

37. Vashi, A. V. et al. Adipose differentiation of bone marrow-derived mesenchymal stem cells using Pluronic F-127 hydrogel in vitro. *Biomaterials* **29**, 573–579 (2008).

38. Cochis, A. et al. Bioreactor mechanically guided 3D mesenchymal stem cell chondrogenesis using a biocompatible novel thermo-reversible methylcellulose-based hydrogel. *Sci. Rep.* **7**, 45018 (2017).

39. Gistelinck, C. et al. Zebrafish collagen type I: molecular and biochemical characterization of the major structural protein in bone and skin. *Sci. Rep.* **6**, 21540 (2016).

40. Shangguan, D., Zhao, Y., Han, H., Zhao, R. & Liu, G. Derivatization and fluorescence detection of amino acids and peptides with 9-Fluorenylmethyl chloroformate on the surface of a solid adsorbent. *Anal. Chem.* **73**, 2054–2057 (2001).

41. Bag, S. et al. Identification of α-enolase as a prognostic and diagnostic preancer biomarker in oral submucous fibrosis. *J. Clin. Pathol.* **71**, 228–238 (2018).

42. Chai, Y., Wang, G., Fan, L. & Zhao, M. A proteomic analysis of mushroom polysaccharide-treated HepG2 cells. *Sci. Rep.* **6**, 23565 (2016).

43. Brown, S. et al. Identification of a new hominin bone from Denisova Cave, Siberia using collagen fingerprinting and mitochondrial DNA analysis. *Sci. Rep.* **6**, 23559 (2016).

44. Schechter, D., Dormann, H. L., Allis, C. D. & Hake, S. B. Extraction, purification and analysis of histones. *Nat. Protoc.* **2**, 1445–1457 (2007).

45. Kapat, K. et al. Simultaneous hydrothermal bioactivation with nanotopographic modulation of porous titanium alloys towards enhanced osteogenic and antimicrobial responses. *J. Mater. Chem. B* **6**, 2877–2893 (2018).

46. Kapat, K. et al. Osteochondral defects healing using extracellular matrix mimetic phosphate/sulfate decorated GAGsagarose gel and quantitative micro-CT evaluation. *ACS Biomater. Sci. Eng.* https://doi.org/10.1021/acsbiomaterials.8b00253 (2018).

47. Kapat, K. et al. Influence of porosity and pore-size distribution in Ti6Al4V foam on physioengineermental properties, osteogenesis, and quantitative validation of bone ingrowth by micro-computed tomography. *ACS Appl. Mater. Interfaces* **9**, 39235–39248 (2017).

48. Rameshbabu, A. P. et al. Investigating the potential of human placenta-derived extracellular matrix sponges coupled with anionic membrane-derived stem cells for osteochondral tissue engineering. *J. Mater. Chem. B* **4**, 613–625 (2016).

49. Perez-Riverol, Y. et al. The PRIDE database and related tools and resources in 2019: improving support for quantification data. *Nucleic Acids Res.* **47**, D442–D450 (2019).

**Acknowledgements**

The authors would like to acknowledge the Central Research Facility (CRF) of the Indian Institute of Technology (IIT), Kharagpur for establishing the MALDI facility. P.P.M thanks to Indian Council of Medical Research (ICMR, Govt. of India), DD, SG, KD thanks to IIT Kharagpur for individual fellowships. The authors acknowledge Mr. Bidhan Chandra Sing for helping in MALDI data acquisition, Krishnabrata Panda and Nantu Dora for technical assistance.

**Author contributions**

P.P.M. and D.D. designed the experiments; P.P.M., D.D., S.G., K.K., K.D. performed experiments, analyzed data; P.P.M., D.D., S.G. drafted the manuscript; R.S. provided technical assistance for HPLC; P.D., N.C.D., A.R.C., A.K.D. and S.D. evaluated the results and corrected the manuscript; All authors reviewed the results and approved the final version of the manuscript.

**Additional information**

**Supplementary information** accompanies this paper at https://doi.org/10.1038/s42003-019-0394-6.

**Competing interests:** The authors declare no competing interests.

**Reprints and permission** information is available online at http://npg.nature.com/reprintsandpermissions/

**Publisher’s note:** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

---

© The Author(s) 2019