Sustainable Dissolution of Collagen and Formation of Polypeptides in Deep Eutectic Solvents for Use as Antibacterial Agents

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Supporting Information

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Annexure S1:

List of advantages our method/technique has over the existing approaches reported in the literature for the dissolving of collagen in DESs and the formation of collagen or collagen peptides.

S. No	Merits of our method	Description of Our Method	Description of Reported Methods
1.	Prevention (Less Waste)	**Our method:** There are three steps involved in the dissolution of collagen in DESs. Step 1: Preparation of DESs i.e. choline chloride:lactic acid (ChCl:LA) and ethylene glycol:zinc chloride (EG:$ZnCl_2$) produce no waste as a side-product. Step 2: Dissolution of collagen takes place by simply adding the collagen in DESs and heating them at optimum temperature conditions. The dissolved collagen is regenerated from DESs using ethanol as an antisolvent. On regeneration, the dissolved collagen resulted in the formation of collagen-like structure/collagen peptides only, without formation of any other non-collagenous material. Therefore, the whole process produces no waste in the environment.	**Reported Methods**: Volatile Organic Solvents (VOSs)[1] and inorganic solvents[2] have been used for the dissolution of collagen producing toxic fumes in the environment. Imidazolium-based ionic liquids (ILs) have also been used for the dissolution of collagen,[3] but they give side products as waste during their synthesis. DESs have also been used to extract collagen peptides from raw material, but with low percentage purity due to the presence of non-collagenous material along with nucleic acid and other proteins in the material. Also, essential and vital amino acids are lost during the extraction process.[4a,b]
2.	Atom Economy	As the atoms needed to prepare DESs are also included in the final product, therefore, the process can be classified as "atom economy	VOSs and inorganic solvents which are employed for dissolving collagen produce waste at the molecular level during their synthesis.

		process." As a result, the preparation of DESs reduces waste at the molecular level. The dissolution and regeneration step produces no side products; collagen gets converted to collagen peptides on dissolution in DESs.	Purifications and several steps are involved in the multi-step process of synthesizing ILs,[5] which are utilized to dissolve or extract collagen. The likelihood of an atom economy loss is therefore high.
3.	Less Hazardous Chemical Syntheses	Components of DESs, the native collagen, and regenerated material are chemically safe. Also, for the preparation of DES, the process is less hazardous as they are formed by simply mixing two components and heating them at ambient temperature.	VOSs and inorganic solvents used for the dissolution of collagen are toxic and therefore, hazardous in nature. Besides this, imidazolium-based ILs which are used for the dissolution of collagen may require high temperature for synthesis.[6]
4.	Design for Energy Efficiency (lower energy inputs, temp and pressure)	The DES has been prepared by simply heating the components at ambient temperature conditions (60 ^{o}C for 6-8 hours). Further, the dissolution has also been carried out at optimum temperatures i.e. 45 ^{o}C, 70 ^{o}C and 90 ^{o}C. At last, regeneration using ethanol has also been carried out at 4 ^{o}C. Therefore, in the whole process, the consumption of energy is minimal. Also, the preparation of DESs and dissolution of collagen occurs at atmospheric pressure.	The dissolution of collagen in VOSs and inorganic solvents usually takes place at high-temperature conditions. Also, multiple steps are involved in the preparation of collagen peptides, which requires energy.[7]
5.	Safer Solvents and	Components used for the preparation of DESs	VOS's and inorganic solvents used for

	Auxiliaries	are non-toxic and biodegradable. Regeneration of dissolved collagen is again a safer step. Therefore, the whole process is safe, and environmentally friendly.	dissolution of collagen are toxic and volatile. Most of the reported ILs used for the dissolution of collagen are non-biodegradable and toxic.
6.	Inherently Safer Chemistry for Accident Prevention (Ease in preparation)	Preparation of DESs also takes place by just mixing and heating the components at 60 ^{o}C-80 ^{o}C for 6-8 hours without the involvement of heavy equipments. The whole process reduces the chances of accident. Further, no other flamable solvent has been used during the synthesis of DES, which again eradicates the risk of flammability during their preparation. The dissolution process of collagen is carried out at three temperatures i.e. 45 ^{o}C, 70 ^{o}C and 90 ^{o}C using simple stirring, which reduced the chance of accident. The regeneration has been carried out at 4 ^{o}C.	VOSs and inorganic solvents used for dissolving collagen are flammable and prone to accident. The components used for synthesis of ILs are toxic which could be dangerous. Also, during the synthesis of many of ILs, greater number of steps are involved which increases the chance of accident.
7.	Design for Degradation (Biodegradability)	Biodegradable and safer components are utilized in the preparation of DES. Ethanol used in the regeneration process and for removing DES from dissolved material is	VOSs, inorganic solvents and ILs used in the dissolution of collagen are non-biodegradable as they persist in the environment.

		also green and environment-friendly.	
8.	Recyclability	Regenerated material in the form of collagen or collagen peptides are further used as an antimicrobial agent.	VOSs and inorganic solvents used in the dissolution of collagen are for one-time used and can't be recycled. Moreover, there is no way to recycle or repurpose the solvents used to prepare collagen peptides.[4,7-9]
9.	Reuse	We reused the used DES and there is no alternation in the structure of collagen or collagen peptides recycled from recycled DESs.	The VOSs and inorganic solvents cannot be reused. Moreover, the DESs used to extract collagen peptides cannot be reused.[4a,b]

Annexure S2:

Molecular weight determination is done using SDS-PAGE: The native collagen and the regenerated material obtained from different DESs (10 mg) were added to 2x Laemmli sample buffer solution (1 ml) of pH 6.8, containing 62.5 mM Tris–HCl, 25% glycerol, 5% *β*-mercaptoethanol, 2% SDS, 0.01% bromophenol blue. Firstly, the samples were vortexed and then shaked in orbital shaker for 1 h at 150 rpm at 45 ^{o}C to disperse the proteins. Later, the samples were heated for 5 min at 100 ^{o}C and centrifuged at 11000xg for 20 min.

SDS-PAGE of native and regenerated collagen obtained from different DESs was performed by loading 10µl of prepared sample supernatant in each well and resolved in 8% resolving gel at a constant current of 25 mA (Mini-Protean Tetra Cell, Bio-Rad Laboratories, Hercules, USA). The loaded sample (tracking dye) move from top of the gel to bottom in the resolving gel and later the gel was removed. The gel was stained overnight with a staining solution having composition of 0.1% Coomassie Brilliant Blue-R250 in 40% methanol and 10% acetic acid in deionized water. The gel was then destained to visualize the bands using 25% methanol and 10% acetic acid in deionized water.

The broad range molecular marker (GeNei, Bangalore, India) containing myosin (205 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (66.0 kDa), ovalbumin (44.0 kDa), carbonic anhydrase (29.00 kDa), soyabean trypsin inhibitor (20.10 kDa), lysozyme (14.30 kDa), aprotinin (6.50 kDa) and insulin (3.50 kDa) were used for estimation of molecular weight of native collagen and regenerated material. The quantification of destained gel was then accomplished by using Bio-Rad EZ Imager (Bio-Rad Laboratories, Hercules, USA).

Table S1: Changes in triple-helical structure of regenerated material in comparison to native collagen.

	Triple-Helical (1654-1660 cm^{-1})	Random Coil (1630-1640 cm^{-1})	β-Sheet (1610-1617 cm^{-1})
	Area of Band		
Native Collagen	75.18	15.718	8.6323
R1-(45)	61.43	23.97	14.74
R2-(45)	61.00	28.24	11.03
R1-(45)-A	58.54	28.93	12.29
R2-(45)-A	57.89	28.53	13.32
R1-(70)	61.18	24.90	11.66
R2-(70)	60.26	29.89	10.03
R1-(70)-A (Water insoluble)	56.26	31.38	11.782
R2-(70)-A (Water insoluble)	52.57	50.07	11.26
R1-(70)-A (Water soluble)	46.22	39.389	15.92
R2-(70)-A (Water soluble)	41.58	40.13	17.06
R1-(90)	38.173	44.63	6.9084
R2-(90)	28.07	54.07	16.52
R1-(90)-A	20.86	54.92	22.78
R2-(90)-A	19.43	57.08	21.84

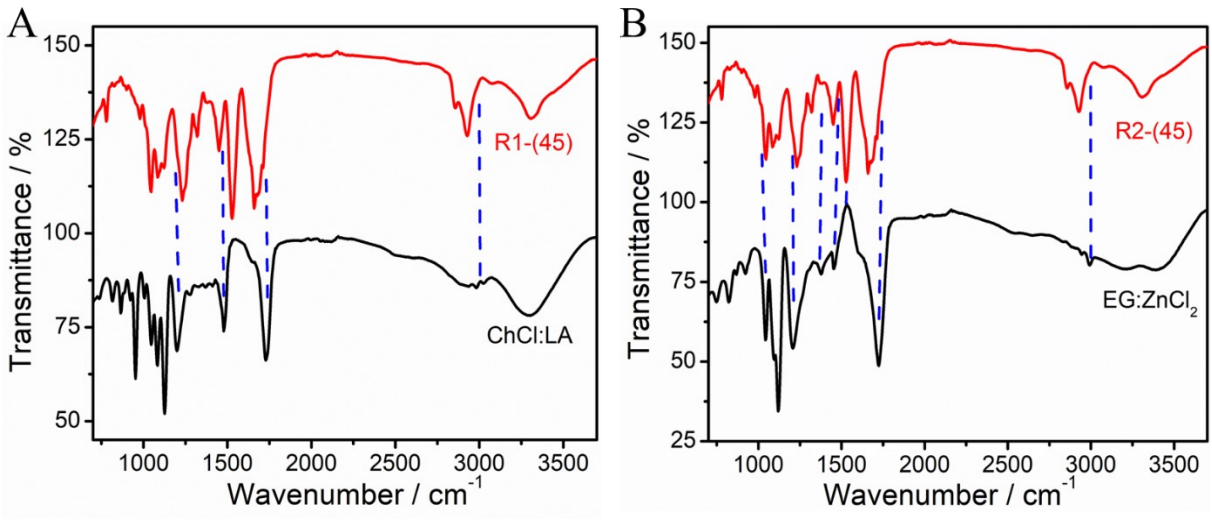

Figure S1: (A) FTIR spectra of native material regenerated from ChCl:LA and (B) EG:$ZnCl_2$ in comparison to DESs respectively.

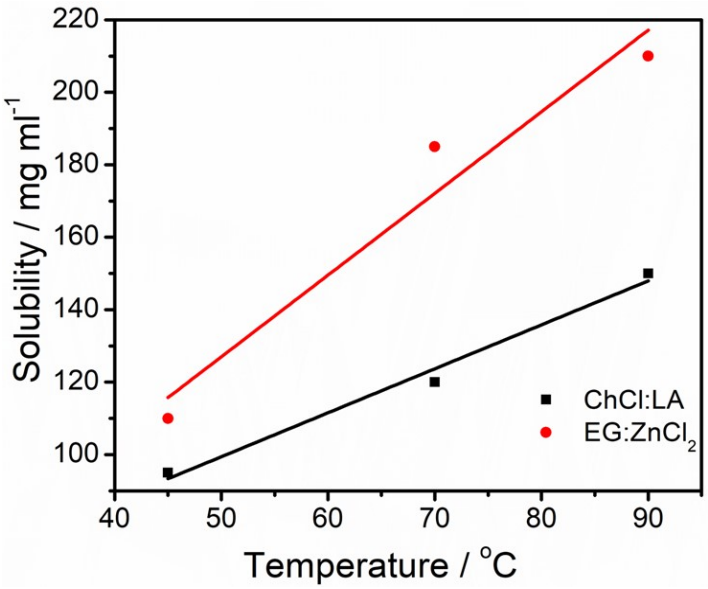

Figure S2: Solubility trend of collagen fibrils in ChCl:LA and EG:$ZnCl_2$ DES.

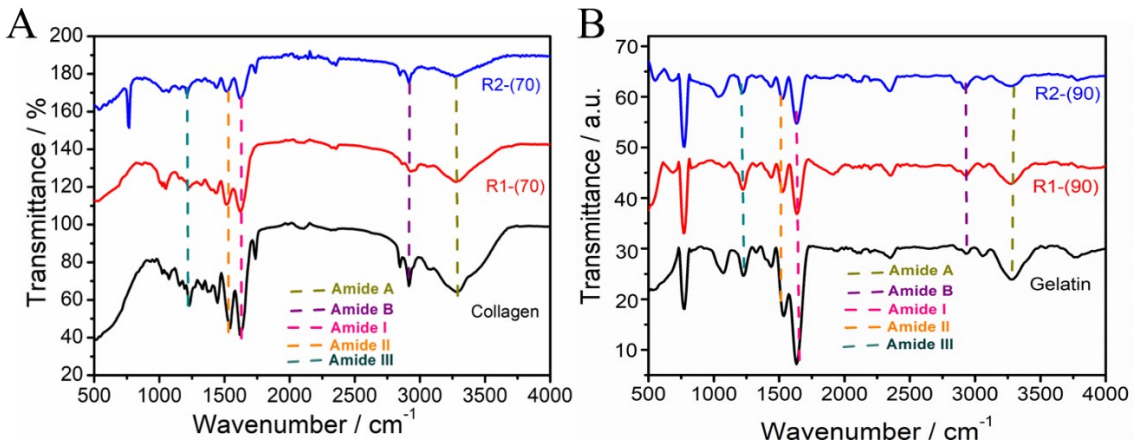

Figure S3: (A) FTIR spectra of native collagen and material regenerated from ChCl:LA; and (B) FTIR spectra of material regenerated from EG:$ZnCl_2$ in comparison to gelatin respectively.

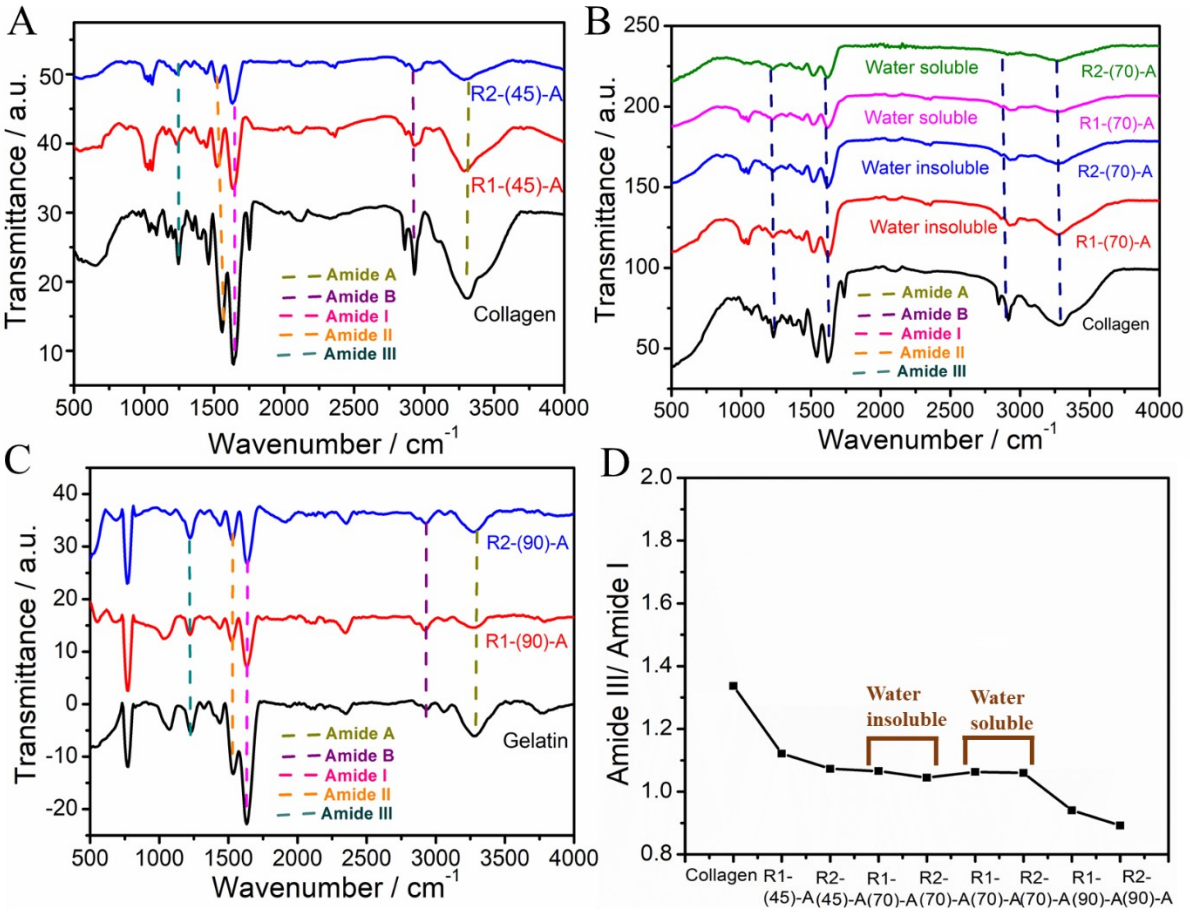

Figure S4: (A) FTIR spectra of native collagen and samples regenerated under acidic conditions from ChCl:LA and EG:$ZnCl_2$; (B) FTIR spectra of water insoluble and water soluble regenerated material respectively; (C) FTIR spectra of material regenerated from DESs under

acidic conditions in comparison to gelatin; and (D) amide III/amide I ratio of native collagen and material regenerated from DESs under different reaction conditions.

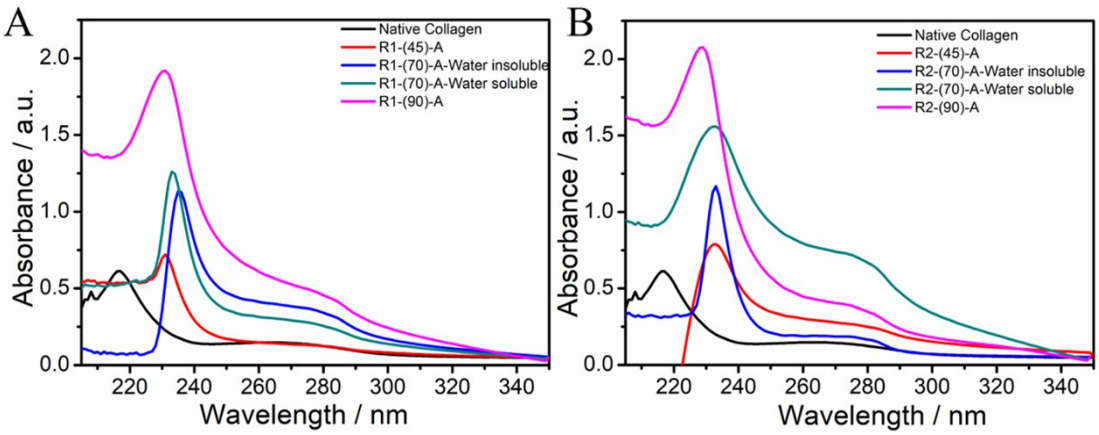

Figure S5: (A-B) UV-vis spectra of native collagen and material regenerated from DESs under acidic conditions.

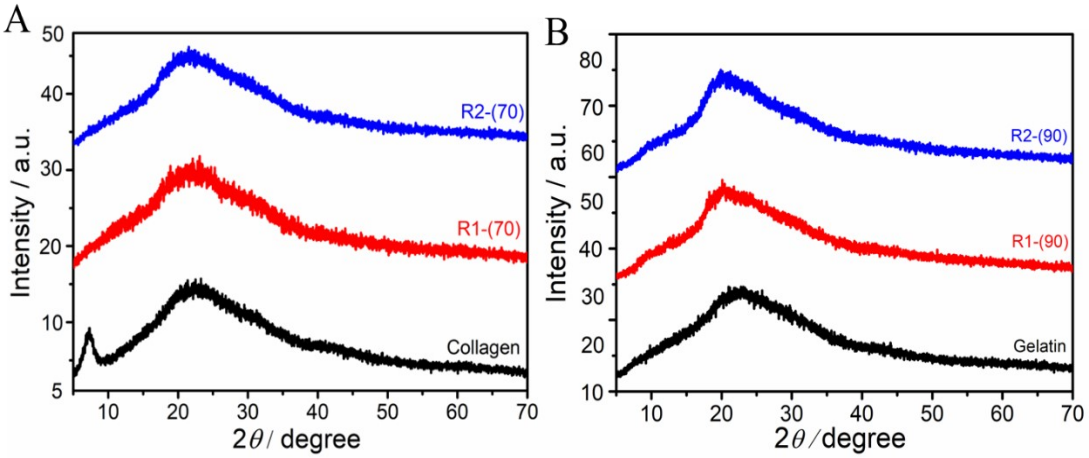

Figure S6: (A-B) X-ray diffraction pattern of material regenerated in comparison to native collagen and gelatin respectively.

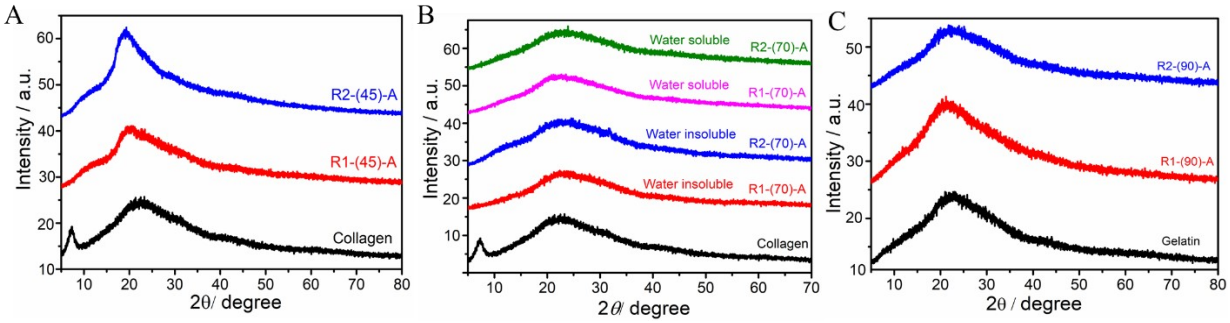

Figure S7: (A-C) X-ray diffraction pattern of material regenerated from DESs under different temperature and acidic condition in comparison to native collagen and gelatin.

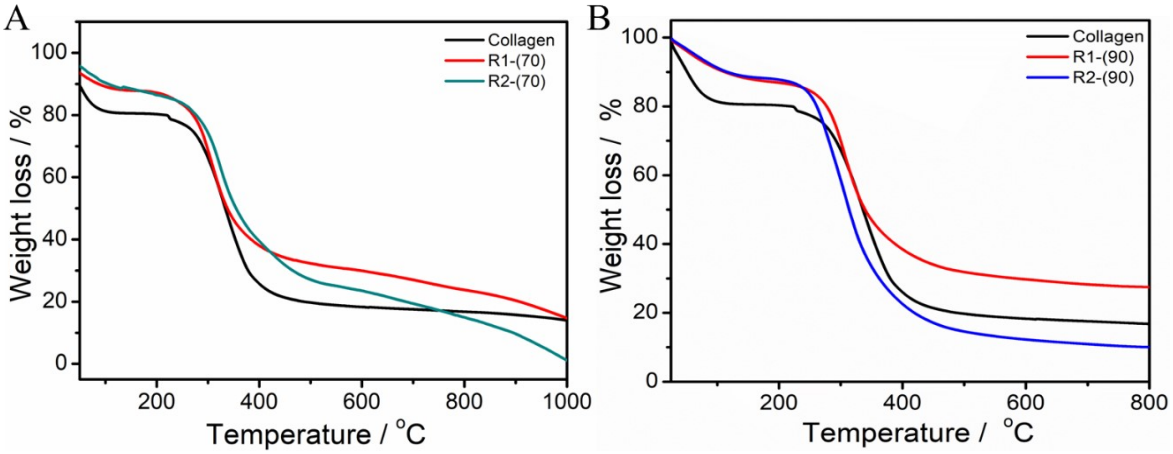

Figure S8: (A-B) TGA profile of regenerated material in comparison to native collagen respectively.

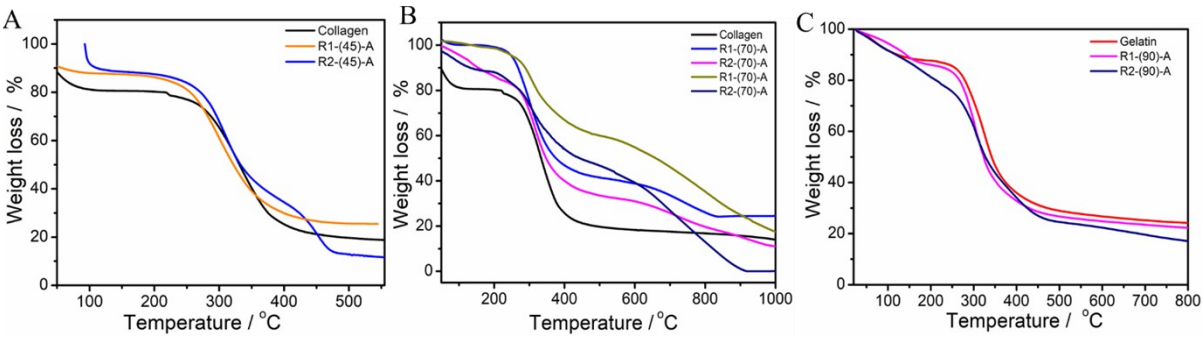

Figure S9: (A and B) TGA profile of water insoluble and soluble regenerated material in comparison to collagen; and (C) TGA profile of gelatin and water soluble regenerated material.

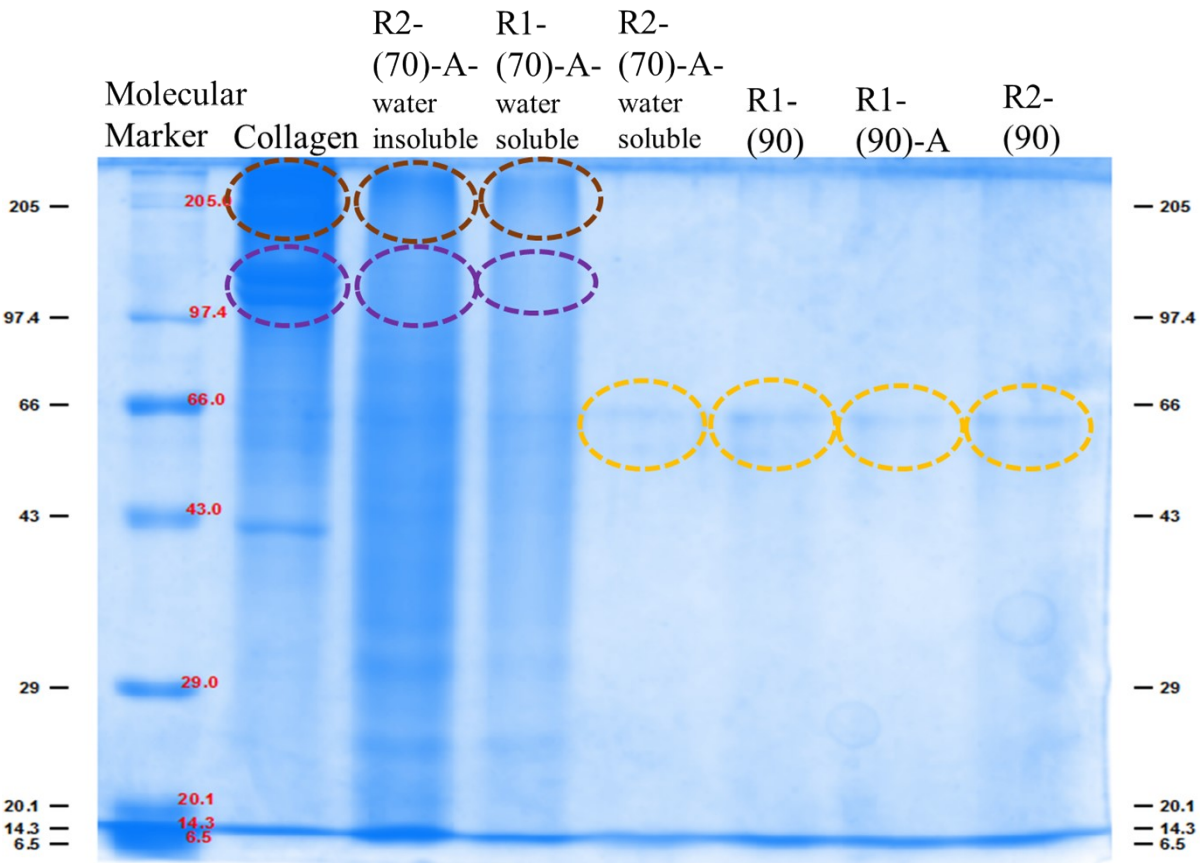

Figure S10: SDS-PAGE displaying the molecular weight of marker used, native collagen and regenerated material under different reaction conditions.

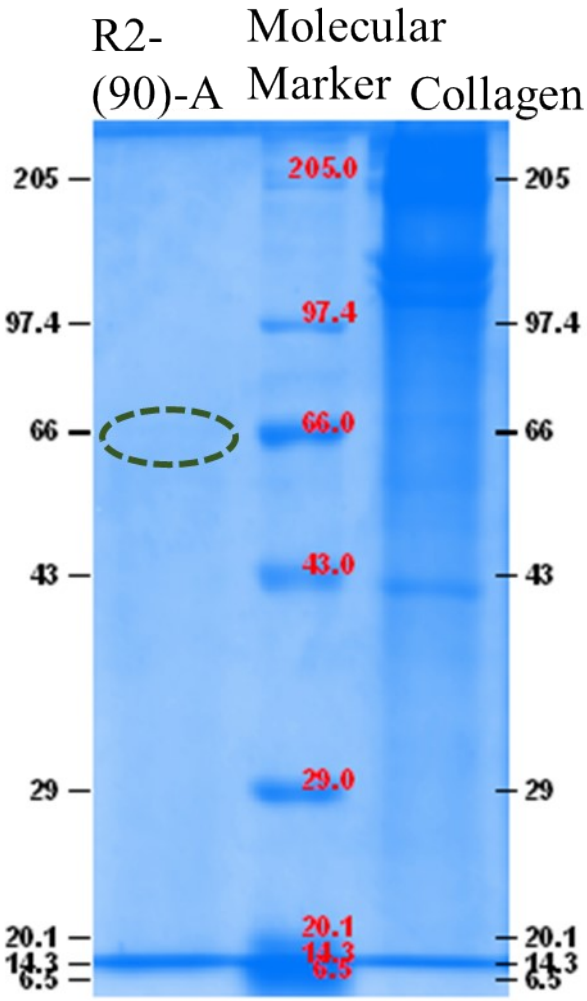

Figure S11: SDS-PAGE displaying the molecular weight of marker used, native collagen and regenerated collagen; lane 1: regenerated material R2-(90)-A.

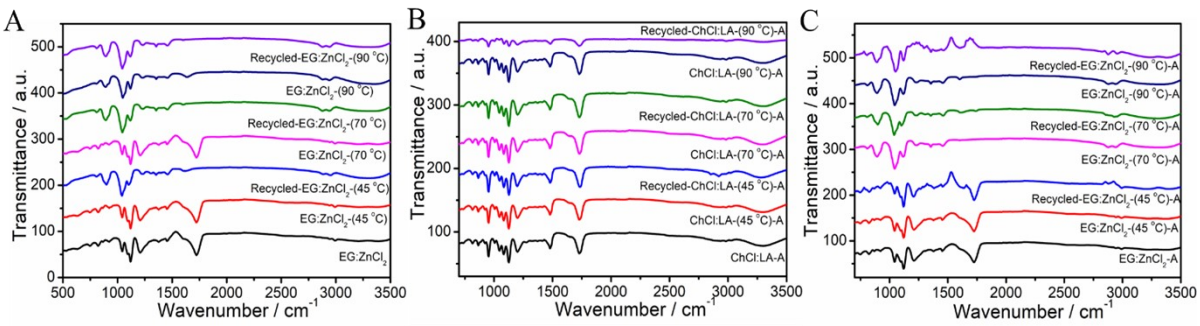

Figure S12: (A) FTIR spectra of recycled EG:$ZnCl_2$ DES at different temperatures in comparison to native DES; (B-C) FTIR spectra of recycled ChCl:LA and EG:$ZnCl_2$ DES in presence of $HCl_{(aq.)}$ at different temperatures in comparison to native DES.

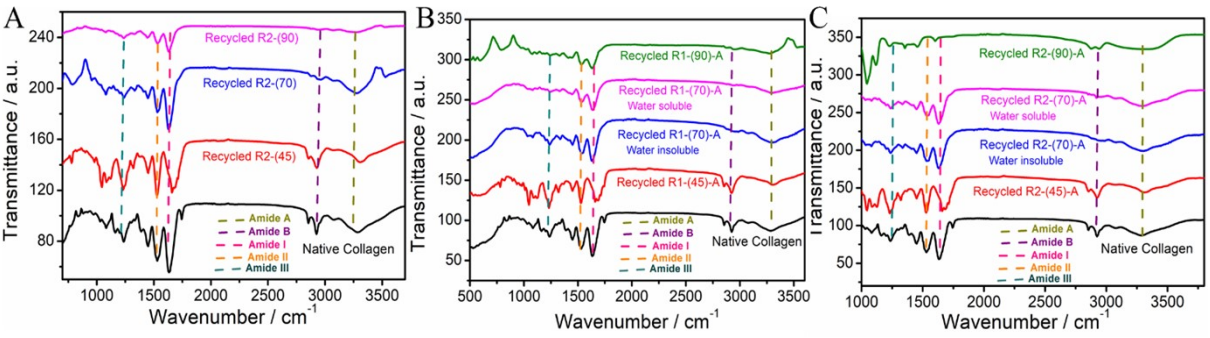

Figure S13: (A) FTIR spectra of material regenerated from recycled EG:$ZnCl_2$ DES at different temperature conditions; and (B-C) FTIR spectra of material regenerated from recycled ChCl:LA and EG:$ZnCl_2$ DES in acidic and different temperature conditions in comparison to native collagen.

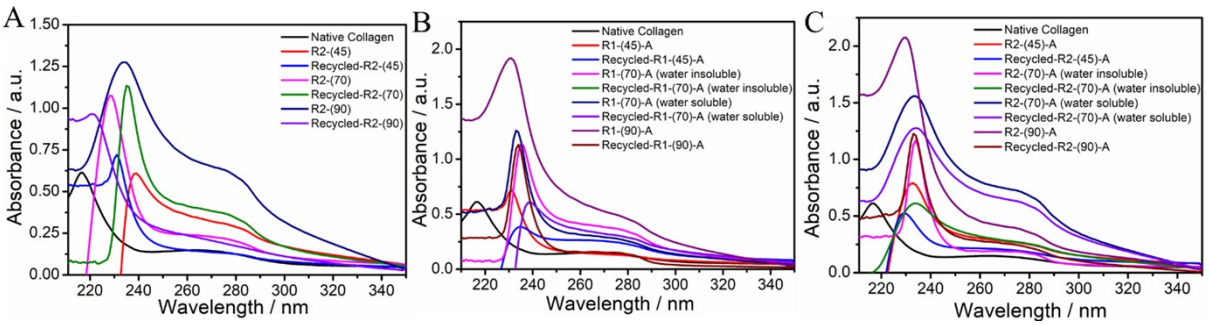

Figure S14: (A) UV-vis spectra of material regenerated from recycled EG:$ZnCl_2$ DES at different temperature conditions; and (B-C) UV-vis spectra of material regenerated from recycled ChCl:LA and EG:$ZnCl_2$ DES in acidic and different temperature conditions in comparison to native collagen.

Table S2: Molecular weight of material regenerated from DESs at various conditions.

45 °C	
R1-(45)	144.8, 133.1, 81.3, 74.0, 44.4, 37.0, 32.3, 27.0, 19.1 and 13.3 kDa
R1-(45)-A	147.9, 133.1, 113.2, 98.3, 83.7, 74.2, 46.7, 38.6, 32.8, 28.1, 19.8 and 13.4 kDa
R2-(45)	154.2, 139.8, 107.8, 89.8, 74.8, 51.5, 43.5, 31.9, 24.6, 19.2, 15.3 and 12.9 kDa
R2-(45)-A	156.4, 141.8, 108.5, 98.8, 71.2, 51.9, 44.1, 33.1, 25.7, 19.1, 15.9 and 12.9 kDa
70 °C	
R1-(70)	152.1, 136.9, 102.6, 76.4, 65.9, 44.4, 38.9, 33.1, 28.3, 25.5. 20.4 and 13.4 kDa
R1-(70)-A (water insoluble)	150.0, 133.1, 84.8, 75.3, 65.0, 47.7, 39.2, 32.2, 28.9, 26.2, 21.7 and 13.7 kDa
R2-(70)	121.2, 95.8, 75.8, 54.8, 51.5, 44.5, 33.2, 25.1, 20.2 and 13.4 kDa
R2-(70)-A (water insoluble)	139.6, 99.9, 86.9, 75.8, 63.1, 49.2, 43.4, 36.3, 32.0, 29.8, 26.8 and 20.9 kDa
R1-(70)-A (water soluble)	140.9, 123.9, 63.4, 55.6, 49.2, 44.3, 40.5, 32.0, 29.9, 28.4, 26.5 and 20.5 kDa
R2-(70)-A (water soluble)	63.9 and 56.2 kDa
90 °C	
R1-(90)	63.4 and 55.0 kDa
R1-(90)-A	62.8 and 55.0 kDa
R2-(90)	62.8 and 54.8 kDa
R2-(90)-A	67.2 and 53.9 kDa

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