Supplementary file

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Proton NMR spectra of engineered gelatin

^{1}H NMR spectra of gelatin, PEG, GEG, PA and GA was recorded at an operating frequency of 500 MHz in a JEOL ECA-500 FT NMR spectrometer. All samples were dissolved in 750µl D_2O individually and ^{1}H- spectra were recorded at 45 ºC (for better solubility) without suppressing the water signal.

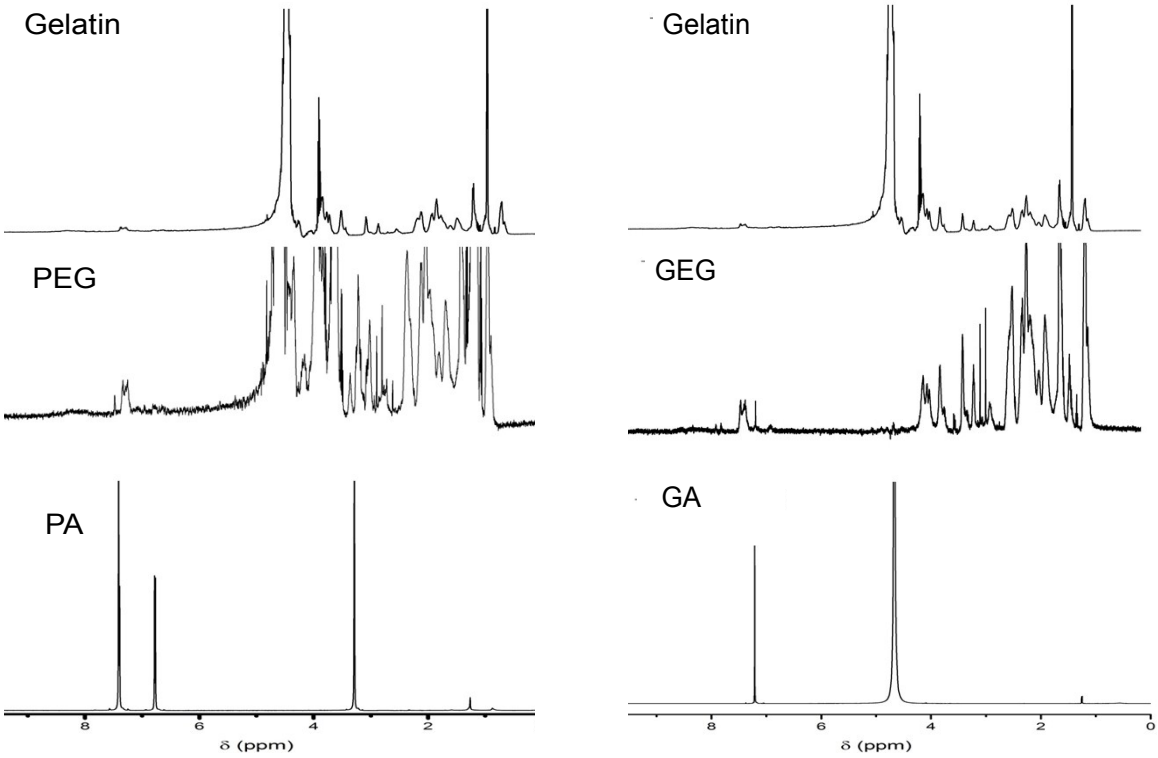

Figure S1 : Proton NMR spectra of Gelatin, PEG, GEG , PA and GA

To confirm the functionalization/modification of gelatin protein, ^{1}H NMR spectrum was recorded for GA, PA, gelatin and engineered gelatin (PEG & GEG) samples. ^{1}H NMR spectrum of PA and GA displayed chemical shifts at δ 7.4 (d, H4) and δ 7.1 (s, H2) respectively. The ^{1}H NMR spectrum of gelatin, and the functionalized gelatin displayed a broad chemical shifts from 7.2 to 7.4 corresponds to residual aromatic amino acid present in the protein backbone. However, PEG and GEG samples shown significant change in the chemical shift values at 7.4, and 7.1 ppm which corroborates with the spectrum of PA and GA.

Contact angle measurements of engineered gelatin

Contact angle measurements made using thin film of gelatin, PEG and GEG samples displayed theta value (θ°) of 82.7 ± 2.3, 66.2 ± 0.8 and 36.15 ± 1.6 respectively as the angle of wettability. In general, when the observed contact angle values are less than 90°, the wettability of the surfaces is high. In the present study, the theta values less than 90° implied good wettability of both PA and GA engineered surfaces and add value to the conjugated protein for its interaction with tissue surfaces when indented to the body system under live condition. It has been expected that the engineering of PA and GA to the gelatin backbone will increase the number of hydroxyl groups and eventually decrease the angle of wettability when compared to gelatin. However GEG shows a 50 % decrease and PEG shows 15 % increase in the theta value when compared to gelatin. This could be attributed to the variation in the solvent activity coefficient of PA and GA. The solvent activity coefficient of PA and GA was found to be 0.11 and 0.002 respectively and in general, higher the solvent activity lower is the solubility and vice versa [1]. Thus, PEG displayed an increase in contact angle compared to gelatin and GEG displayed decrease in contact angle. Similar observations were made by Xi Yang *et al* in the dopamine –coupled human gelatin samples [2].

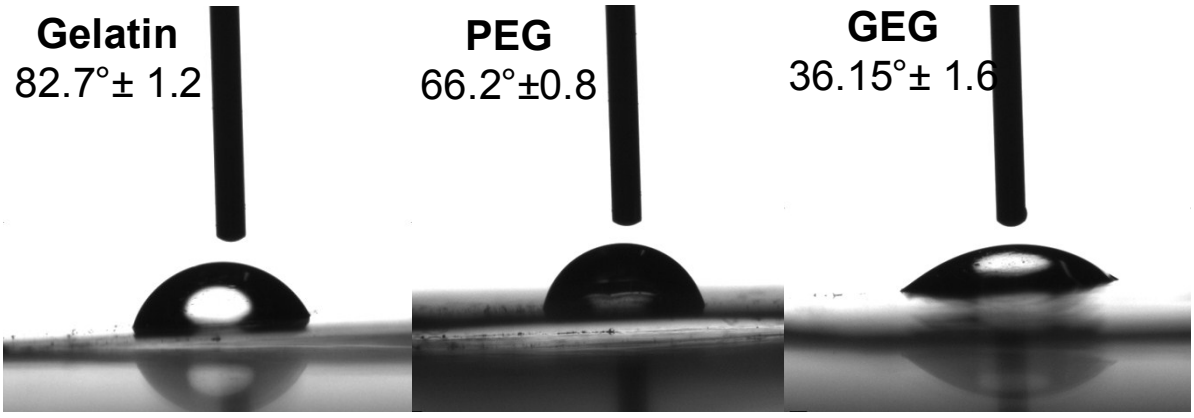

Fig. S2 Contact angle measurements

Rate of swelling and evaporation measurements for PEG-gel and GEG-gel

The swelling property GEG-gel and PEG-gel at different pH was studied by conventional gravimetric procedure. PEG-gel and GEG-gel prepared at 10% concentration of PEG and GEG respectively were pre weighed and immersed in the different pH solutions, viz., 3.0, 6.0, 7.0 and 9.0. Hydrochloric acid (0.05 N) and 0.05 N NaOH was used for the preparation of the test solutions. Periodically the swollen gels were taken and the excess surface water was removed with the help of a filter paper and weighed and the percentage increase in weight was calculated accordingly [3].

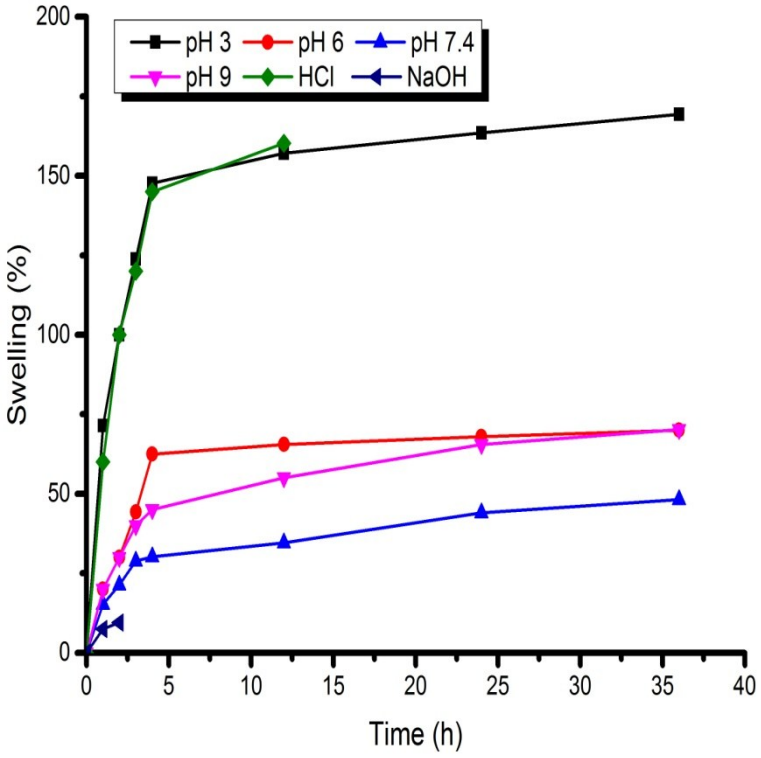

Fig: S3 Rate of swelling of GEG-gel

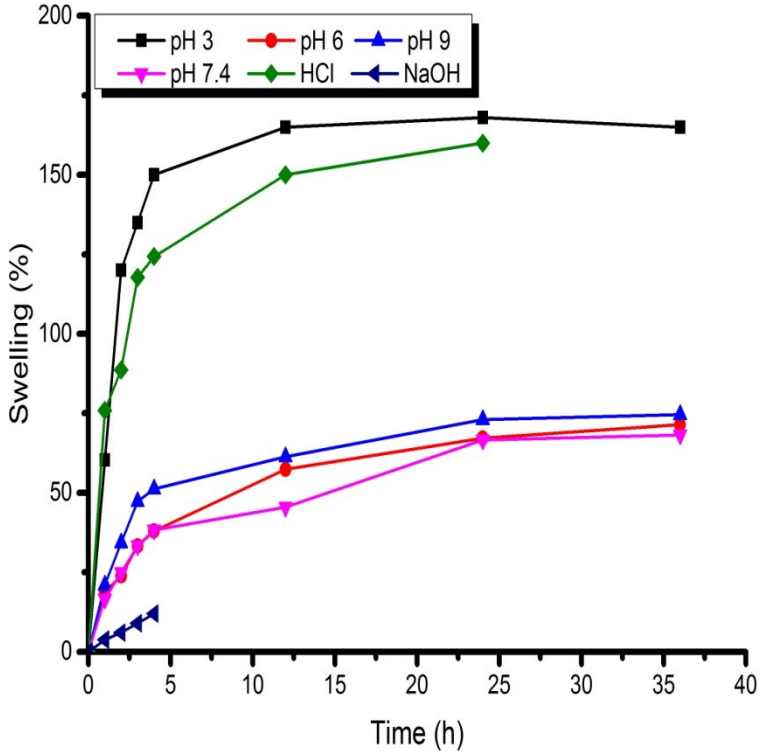

Fig. S4 Rate of swelling of PEG-gel

Swelling study was carried out for PEG-gel and GEG-gel at 10 % (w/v) concentration and as a function of pH (3.0, 6.0, 7.4 and 9.0) at 37 °C for the period of 36 h. Figs. 3c & d depicts the swelling profile of the samples with respect to pH. Both PEG-gel (Fig. S4) and GEG-gel (Fig. S3) attains equilibrium in swelling within 12 h of incubation and shows maximum swelling in acidic environment. At pH 6.0, 7.4 and 9.0, there was no significant difference in the swelling profile of PEG-gel, whereas, GEG-gel displayed less swelling (48.2 %) at pH 7.4.

With regard to the difference in the swelling property, the reason may be attributed to the charge distribution in amino acids. At acidic pH, most of the charged functional groups in amino acid (-COOH, $-NH_2$) gets protonated and exist as overall positive charge of the protein. The net positive charge creates a repulsive force between the gelatin molecules and enhances the swelling to the maximum. In the presence of alkali environment (0.05 N NaOH), the gel swells initially and degrades as the incubation period extended. The alkali pH may hydrolyze the peptide bond of the proteins [4]. There was not much difference in the

swelling ratio of the gelatin gel in the pH range of 6 to 9, the reason may be attributed to the denser crosslinking of the protein and further, the gelatin used in the present study has a isoelectric point of (pI) 7.0 to 9.0 and at this pH, the charge distribution of amino acids will be near to neutral, which does not allow the gel to swell to the maximum.

Rate of evaporation of PEG-gel and GEG-gel

With reference to the rate of evaporation studies, PEG-gel and GEG-gel prepared as described in the previous paragraph were kept at 37 °C and at 35 % relative humidity for the period of 48 h. After regular intervals of time, the weight was measured. Rate of evaporation was calculated in percentage as shown; Rate of evaporation (%) = W_t/W_o X 100; where, W_o and W_t are the initial weight and the weight after time‘t’ respectively.

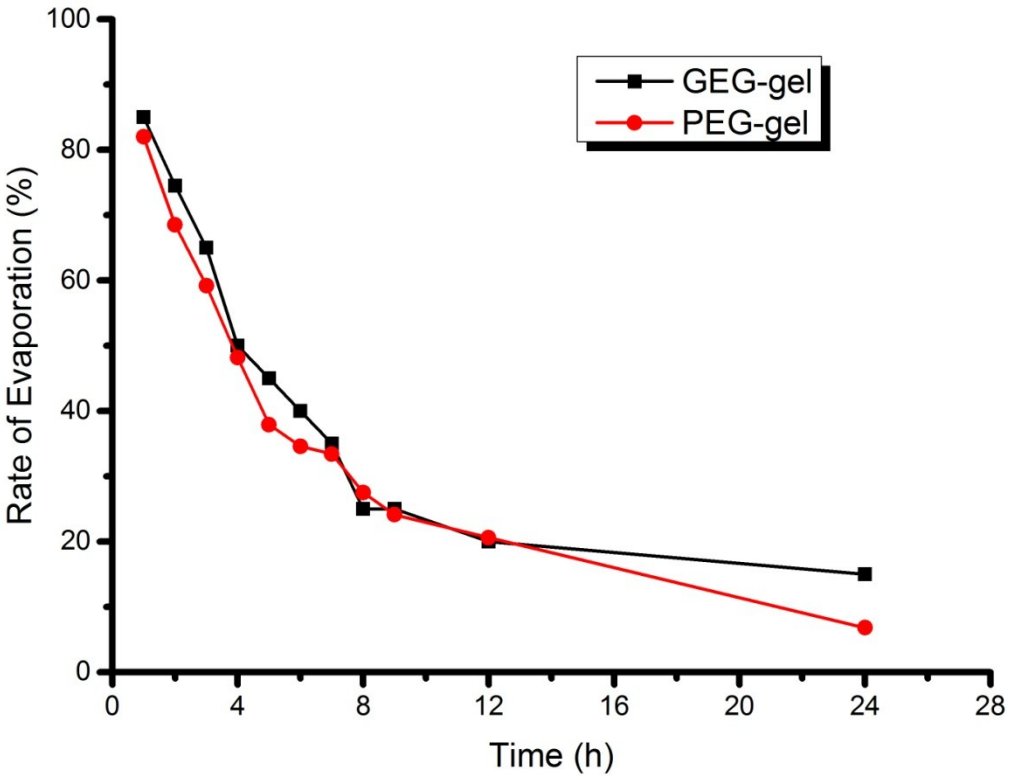

Fig. S5 rate of evaporation of PEG-gel and GEG-gel

The rate of evaporation of PEG-gel and GEG-gel was analyzed at 37 °C and at 30 % humidity (Fig. S5). PEG-gel showed enhanced rate of evaporation compared to GEG-gel. After 24 h, the rate of evaporation was found to be 6.8 and 15 % for PEG-gel and GEG-gel respectively. Similarly, the change in rate of evaporation might be due to the crosslinking density of the engineered gelatin gels. In general, the loss in weight of the hydrogel when exposed to air indicates the promising mobility of the molecules in the hydrogel, which is actually a requisite property when wound healing application of hydrogel is concerned. According to Ganji *et al.,*[5] on theoretical studies on swelling suggested that the size of pores determines the equilibrium swelling and the super porous hydrogels, where, the pores are in the range of several hundred micrometer act as an open channel system swell in aqueous solution to an equilibrium state in a matter of a minute regardless of their size.

Biodegradability assay: *In vitro* assessment

Biodegradability of PEG-gel and GEG-gel was assessed according to the procedure summarized. Ten milligram of oven dried samples of gelatin, GEG, PEG, GEG-gel and PEG-gel were exposed to enzyme buffer at pH 7.5 containing 50 mM Tricine, 10 mM Calcium Chloride and 400 mM Sodium Chloride. Collagenase enzyme was added (5 U/ml) and kept for incubation at 37 ºC. Release of amino acids upon degradation was analyzed using TNBS assay for the period of 48 h [6].

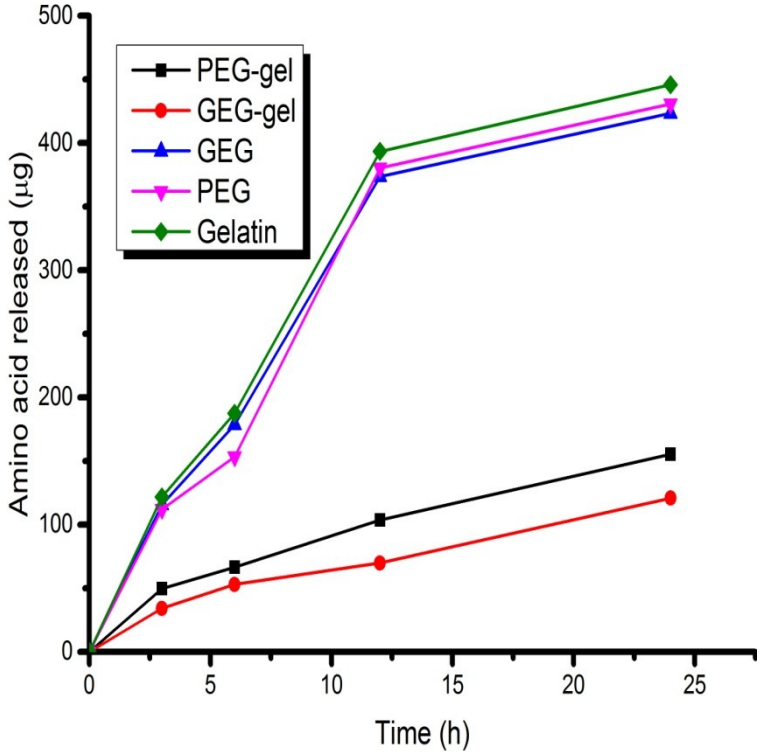

Fig. S6 *in vitro* biodegration of PEG-gel and GEG-gel

In vitro biodegradation studies were performed according to the procedure followed by Raja et al. [7]. Gelatin and both engineered gelatin does not show any significant difference in the enzymatic degradation pattern (Fig. S6). However, upon oxidation, both PEG-gel and GEG-gel shows fourfold decrease in the degradation pattern after 24 h. GEG-gel after 24 h showed lesser degradation (120.8 µg) compared to PEG-gel (155.2 µg), which is an added advantage to the material. The change in the degradation pattern of the engineered gelatin gel might be due to the increased level of crosslinking density, which prevents the exposure of the active cleaving site of the protein towards the collagenase. According to Lutolff *et al*., [8] the controlled swelling and degradation of hydrogels have potential application as drug depot for drug delivery, encapsulating therapeutic proteins and growth factors.

Assessment on Radical scavenging property of engineered proteins

Gelatin, GEG and PEG samples of varied concentrations were subjected to radical scavenging study using DPPH* at room temperature under dark condition [9]. The samples were dissolved in distilled water separately to attain a final concentration of 1 mg/ml. The sample concentrations were varied by taking 50 to 400 µl and made up to 1 ml using 95 % ethanol. To that 125 µl of 0.002 % of DPPH in 99.5 % of ethanol was added. After the scheduled time intervals, a decrease in absorbance was measured at 517 nm. Tests were performed in triplicates and the scavenging effect was calculated as follows:

$$\text{Radical Scavenging Activity (RSA) \%} = (A - B)/B \times 100$$

Where, A, B are absorbance of control and sample respectively. Water without gelatin, PEG, and GEG were taken as control and the sample without DPPH were taken as a blank.

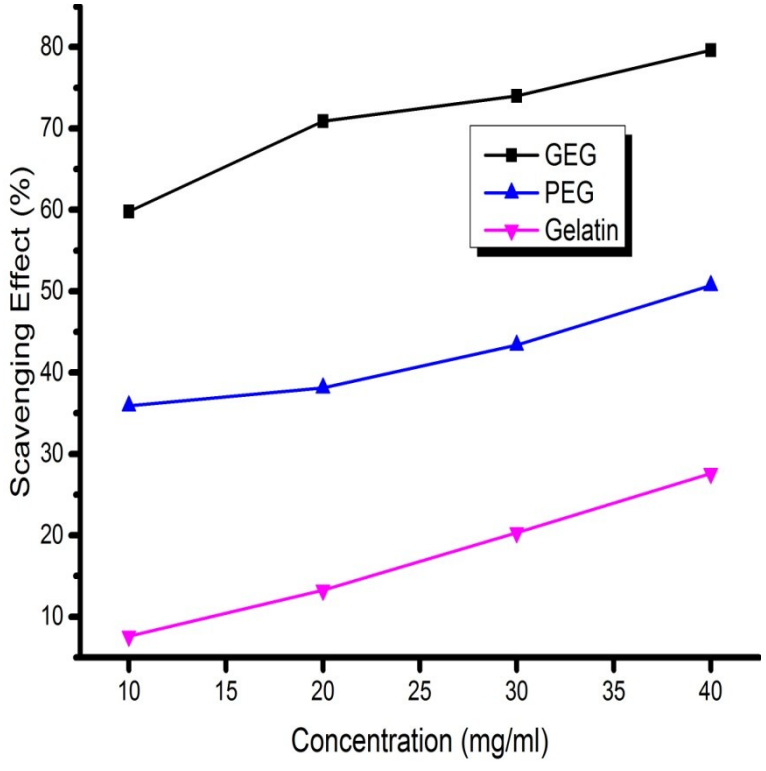

Fig. S7 RSA of PEG, GEG and gelatin

It has been observed that both PEG and GEG samples demonstrated appreciable DPPH free radical scavenging property, but, depends on the concentration of the samples (Fig. S7). A 50 % reduction in the free radical activity of DPPH was observed at 5 and 40

mg/ml concentrations of GEG and PEG respectively. This is an added advantage to the engineered protein. Further increase in the concentration, no proportionate increase in scavenging activity was observed. Precipitation of the samples at higher concentration in the presence of DPPH interferes with the measurements.

Release and accumulation of ROS in the wound imparts deleterious effect in the healing of wound. Management of wounds through antioxidants is always welcomed. Though PA and GA are well known antioxidants, after tethered with gelatin, whether the same property will be exhibited by the samples have been studied using DPPH free radical. According to Albu et al. [10] incorporation of tannic acid to collagen gel increases the antioxidant activity of collagen gel from 55.8 to 71.4 % and has been reasoned to the presence of the number of aromatic hydroxyl groups.

Cell Culturing and maintenance

NIH 3T3 embryonic mouse fibroblast cells procured from NCCS, Pune, India used for the present study. The cultures were maintained in DMEM supplemented with 10% Fetal Bovine Serum (FBS), 200mM Glutamine, 2mg/ml Sodium bicarbonate and 1X antibiotic and antimycotic solution. Periodically the medium was replaced. The cells were cultured in tissue culture flasks and incubated at 37°C in a humidified atmosphere of 5% CO_2. Trypsin at 0.05% was used to detach the cells.

Table S1 : The average mesh size (ξ), average molecular weight between the crosslinks (M_c) and viscoelastic property of the samples PEG-gel & GEG-gel derived from the rheological data.

	Concentration (% w/v)	**G′ (Pa)**	**G″ (Pa)**	**tanδ G″/G′**	**ξ (nm)**	**M_c (kg/mol)**
PEG-gel	8	654	28	0.04	18.4	1.01
	10	889	13.9	0.01	16.6	0.932
	12	1365	43.0	0.03	14.4	0.727
GEG-gel	8	1245	12.6	0.01	15	0.53
	10	1370	13.9	0.01	14.4	0.6
	12	1460	14.2	0.09	14.1	0.681

Incision wound helaing model

In vivo tissue approximation of GEG-gel was assessed by incision wound model. For incision wound, thirty six albino (Wistar strain) rats with an average weight of 200 ± 25 g was segregated into three groups (Group I Control; Group II GEG gel treated; Group III Fibrin sealant treated (positive control)) and twelve animals in each group.

With regard to incision wound study, a 3 cm long paravertibral incision was made using a standard surgical blade. Except group I, all the wounds in group II and group III were approximated using GEG-gel and fibrin sealant. On day 4, 10, and 20, the test animals were euthanized by cervical dislocation and the pelt (6 x 6 cm) was collected for tensile strength measurement and histological studies. Tensile strength of the skin sections (4 X 1 cm perpendicular to the wound surface) were calculated using the peak detachment force obtained from the Instron instrument. For histopathological studies, H&E staining was perfomed. Previously the tissue sections were stored in 10 % buffered formalin for one week. Using phase contrast microscope (Nikon Eclipse 80i), the tissue sections were observed for the reduction in wound area and to measure the collagen synthesis, Masson's trichrome staining was performed and analyzed.

Fig. S8

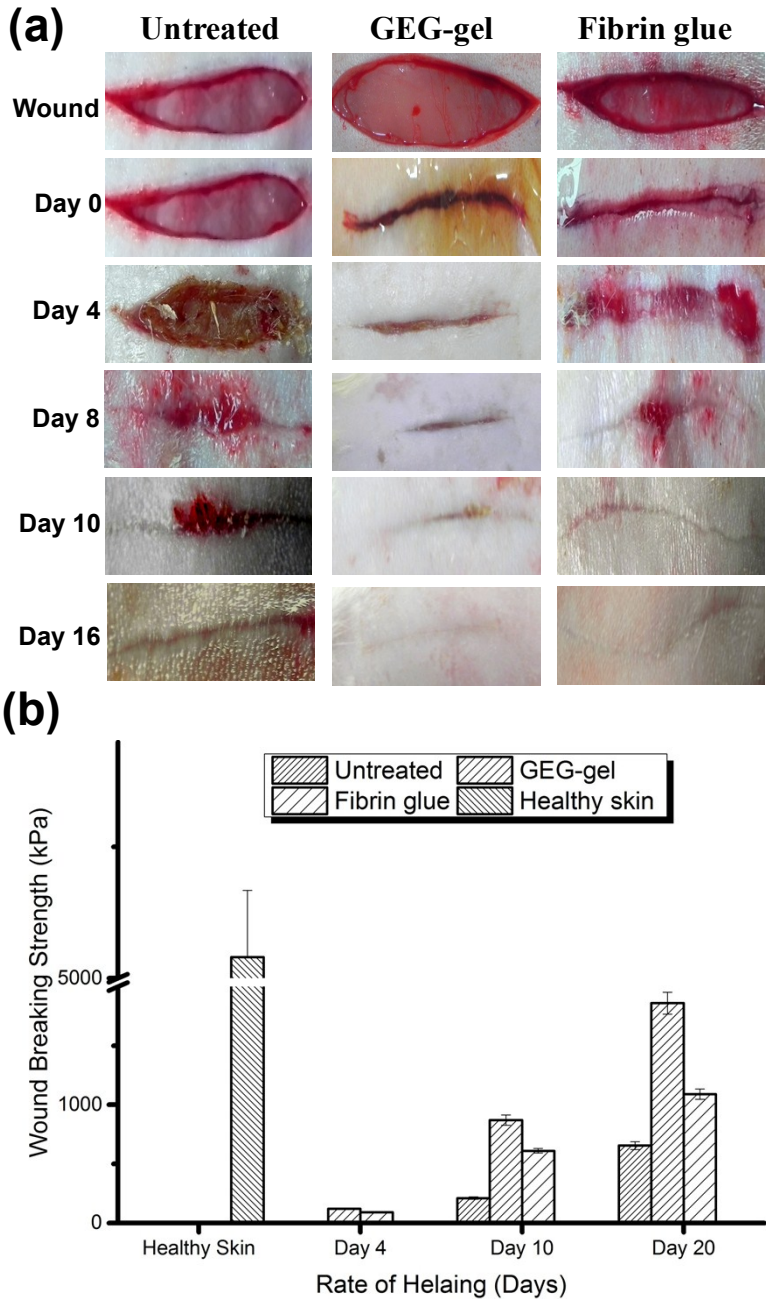
(a)
Untreated
GEG-gel
Fibrin glue
Wound
Day 0
Day 4
Day 8
Day 10
Day 16
(b)
Untreated
GEG-gel
Fibrin glue
Healthy skin
Wound Breaking Strength (kPa)
5000
1000
0
Healthy Skin
Day 4
Day 10
Day 20
Rate of Helaing (Days)

Fig. S8

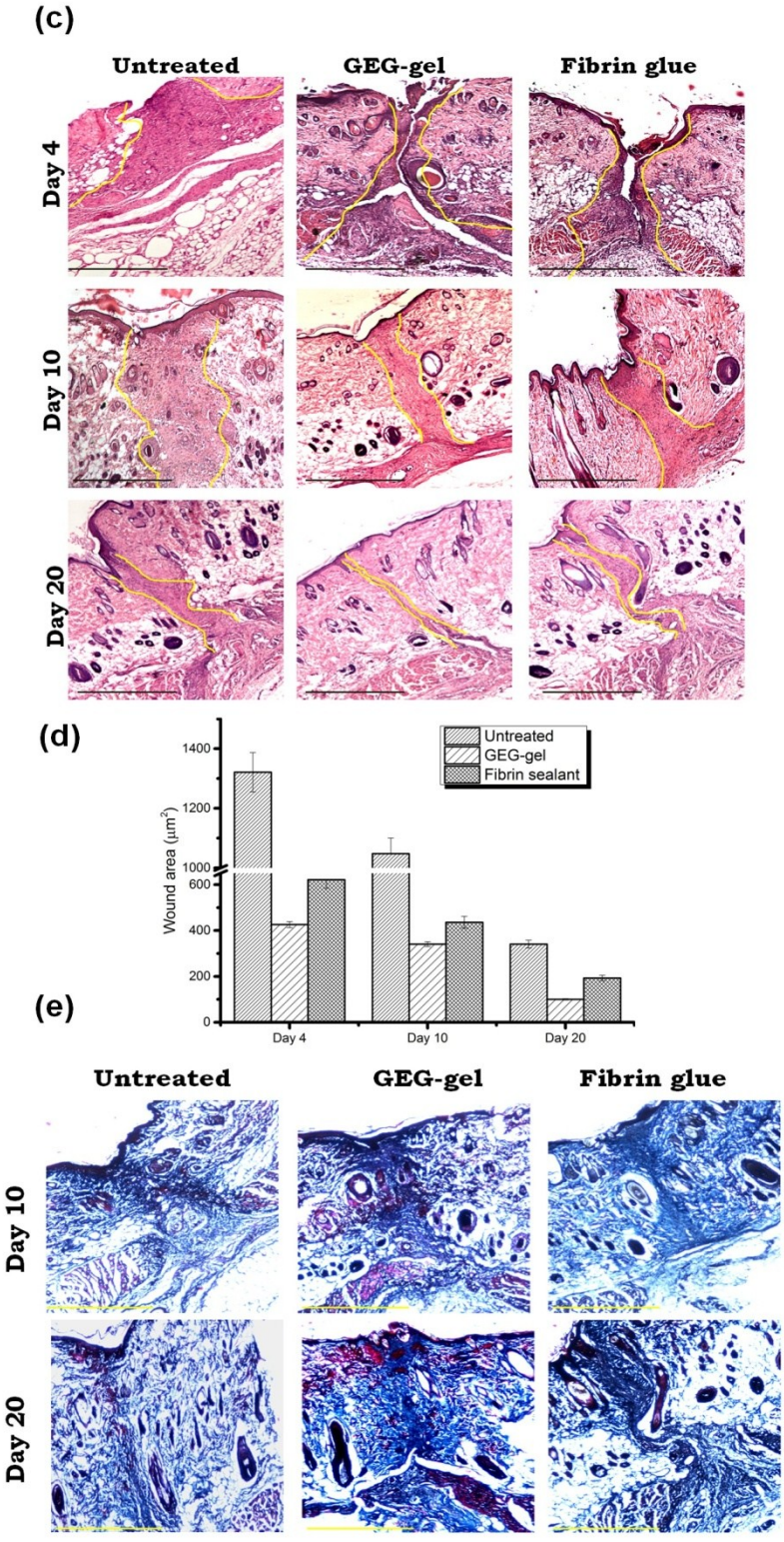

Fig. S8 (a) In-*vivo* incision wound model studies carried out in albino rats Wistar strain demonstrates the tissue approximation property of GEG-gel observed at different time periods and compared to the standard fibrin glue and untreated wound.
(b) Tensile strength of the rat pelt harvested for the experimental samples and at different time intervals

(c) Histo-morphological analysis of rat skin sections using hematoxylin and eosin staining demonstrates the wound healing pattern for the GEG-gel compared with fibrin sealant and untreated wound
(d) Wound area measurements obtained from the H& E staining demonstrates the reduction in wound area (measured in μm^2).Scale bar measures 1 mm.
(e) Masson's trichrome staining of tissue sections of experimental samples. The intense blue colour demonstrates deposition of collagen. The scale bar measures 1 mm.

Wet tissue approximation – *In vivo* Incision wound model

In order to assess the hemostatic tissue adhesive property of the GEG-gel, in vivo incision wound model was performed on the dorsal side of the albino rats. The wound healing period was observed for 20 days (0, 4, 8, 10 and 20) and photographed (Fig. S8a). Sample and fibrin glue does not show significant difference in the healing rate on day 4, however as the day prolongs, on comparing with fibrin glue and untreated wound GEG-gel can able to hold the ends of the incised skin firmly and accelerate the healing. To further elucidate the wound healing efficiency of the GEG-gel, Tensile strength measurements were carried out for the healed skin on different time intervals (day 0, 4, 8, 10 and 20). Native skin shows a tensile strength value of 5100 kPa (Fig. S8b). On day 4 the tensile strength values for all the wounds does not show any significant difference. The skin strength of GEG-gel treated wound shows 870 kPa and 1860 kPa of wound strength on day 10 and 20 respectively, which is comparatively higher than the tensile strength of the fibrin glue and untreated wound.

Histological examination of tissue sections demonstrated that approximation of cut ends starts from the dermis level and significant reduction in the wound area was with GEG-gel compared to fibrin glue and control groups (Fig. S8c). About three fold decrease in the wound area in GEG-gel treated groups compared to control groups. Wound area of 341, 99 and 193 μm^2 was observed respectively for experimental groups, control, GEG-gel and fibrin sealant on day 20 (Fig. 5d). An appreciable amount of collagen deposition (Masson's trichrome staining) in GEG-gel treated tissue sections further substantiates increase in the

secretion of extracellular matrix production. The reason for high tensile strength for GEG-gel treated tissues could be due to the appreciable amount of deposition of collagen during tissue approximation process as evidenced from the staining studies(Fig. S8e).

Comparisons on salient features of phenolic acid tethered gelatin

Table S2 (Supplementary file) depicts the overall comparative assessment made for the phenolic acids engineered protein hydrogel. Though the present study emphasized gallic acid and protocatechuic acid engineered gelatin, our previous results on caffeic acid [7, 11] has also been considered for better understanding on the benefits of phenolic acids tethered gelatin hydrogel. It has been observed that all the three phenolic acids well tethered with gelatin after activation with EDS-NHS. The number of free hydroxyl groups in tethered gelatin determines the various physical and mechanical properties. The less curing time, ie., less than 4 sec shown by GEG reasoned to the presence of three hydroxyl groups. Though CEG and PEG displayed two free hydroxyl groups, but the significant variation ($P < 0.005$) in curing time observed needs explorations. When comparing the gel strength, adhesive strength, swelling ratio, GEG samples ranks first. However, with regard to hemostyptic, adhesive and wound healing properties, no significant difference was observed between CEG and GEG samples.

Table. S2 Comparative assessments of Protocatechuic acid,Gallic acid and Caffeic acid engineered gelatin based injectable hydrogel.

Description	PEG	GEG	CEG [11, 12]
No. Of Aromatic Hydroxyl groups	Two	Three	Two
Gelation time (s) at 10 % (w/v)	60 ± 10	4 ± 1	17 ± 3
Viscosity (cP)	19.2 ± 1.2	10.9 ± 0.7	11.73 ± 0.2
Contact angle (θ°)	74.6 ± 2.3	36.15 ± 1.6	82.79 ± 1.2
Surface charge (meV)	6.4 ± 0.9	5.8 ± 0.9	3.67 ± 0.03
Gel strength (kPa) (rheology)	1080 ± 50	1410 ± 45	1420 ± 20
***In vitro* Adhesive strength (kPa)**	28.3 ± 3.6	35 ± 2.3	16.25 ± 4.5
Swelling ratio at 12 hours in PBS	45.5 ± 2.25	34.6 ± 2.44	56.6 ± 1.49
Rate of evaporation at 37 °C (%)	20 ± 0.6	20.6 ± 0.5	52.2 ± 1.5
***In vitro* biodegradation (µg of amino acid released)**	155.2 ± 7.75	120.8 ± 7.6	10.2 ± 1.5
Radical scavenging activity (%) for 10mg/ml	35.9 ± 1.6	59.8 ± 3.0	30.5 ± 1.52
Bio-compatibility	Compatible	Compatible	Compatible
Hemorrhaging	Hemostyptic	Hemostyptic	Hemostyptic
***In vivo* incision wound**	Not determined	Promotes tissue approximation	Promotes tissue approximation
***In vivo* excision wound**	Not determined	Promote wound healing	Promote wound healing

[1] C.M. Galanakis, V. Goulas, S. Tsakona, V. Gekas, Predicting the solubilization preference of natural phenols to different solvents, 11th International Congress on Engineering and Food, 2011.
[2] X. Yang, L. Zhu, S. Tada, D. Zhou, T. Kitajima, T. Isoshima, Y. Yoshida, M. Nakamura, W. Yan, Y. Ito, Mussel-inspired human gelatin nanocoating for creating biologically adhesive surfaces, International Journal of Nanomedicine, 9 (2014) 2753-2765.
[3] B. Balakrishnan, M. Mohanty, P.R. Umashankar, A. Jayakrishnan, Evaluation of an in situ forming hydrogel wound dressing based on oxidized alginate and gelatin, Biomaterials, 26 (2005) 6335-6342.
[4] Y.J. Wang, Formulation, characterization, and stability of protein drugs, Springer Science & Business Media1996.
[5] F. Ganji, S. Vasheghani-Farahani, E. Vasheghani-Farahani, Theoretical description of hydrogel swelling: a review, Iran Polym J, 19 (2010) 375-398.
[6] R. Fields, Estimation of amino groups using tnbs, Methods Enzymol., 25 (1972) 464-469.
[7] S.T.K. Raja, T. Thiruselvi, R. Aravindhan, A.B. Mandal, A. Gnanamani, In vitro and in vivo assessments of a 3-(3, 4-dihydroxyphenyl)-2-propenoic acid bioconjugated gelatin-based injectable hydrogel for biomedical applications, Journal of Materials Chemistry B, (2015).
[8] M. Lutolf, J. Hubbell, Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering, Nature biotechnology, 23 (2005) 47-55.
[9] J.H. Chen, C.-T. Ho, Antioxidant activities of caffeic acid and its related hydroxycinnamic acid compounds, Journal of Agricultural and Food Chemistry, 45 (1997) 2374-2378.
[10] M.G. Albu, M.V. Ghica, M. Giurginca, V. Trandafir, L. Popa, C. Cotrut, Spectral characteristics and antioxidant properties of tannic acid immobilized on collagen drug-delivery systems, Revista de Chimie–Bucharest, 60 (2009) 666-672.
[11] S. Thirupathi Kumara Raja, T. Thiruselvi, G. Sailakshmi, S. Ganesh, A. Gnanamani, Rejoining of cut wounds by engineered gelatin keratin glue, Biochimica et Biophysica Acta (BBA)-General Subjects, 1830 (2013) 4030-4039.