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# *In vitro* and *in vivo* assessments of 3-(3,4-Dihydroxyphenyl)-2-propenoic acid bioconjugated gelatin based injectable hydrogel for biomedical applications

Thirupathi Kumara Raja S,[1] Thiruselvi T,[1] Aravindhan R,[1]
Asit Baran Mandal,[1] and Gnanamani A [1*]
[1]CSIR-CLRI
Adyar, Chennai
Tamil Nadu, India

* Author for correspondence

Dr. A. Gnanamani
Microbiology Division
CSIR-CLRI
Adyar, Chennai
Tamil Nadu, India
Email: gnanamani3@gmail.com
Fax: No. 91-44-24912150

**Abstract**

Imparting functional properties to a biomaterial for high end application is always a challenging task. In the present study, an attempt was made to construct an injectable hydrogel through bioconjugation of di-hydroxy phenolic acids to gelatin backbone. Bioconjugating caffeic acid with gelatin followed by oxidation with mild oxidation agents provide a hydrogel with all the requisite properties (biocompatibility, controlled biodegradability, antioxidant, antimicrobial and wound healing). Bioconjugation was performed using EDC/NHS and the resultant gel named as Caffeic acid Bioconjugated Gel (CBG gel). The physico-chemical, rheological, swelling, *in vitro* studies (biocompatibility, biodegradability, antimicrobial property, antioxidant property, drug release properties) and *in vivo* studies (biocompatibility, biodegradability, and wound healing) of the CBG gel was carried out using standard protocols. The bioconjugation was confirmed by $^{1}$H NMR and UV-Vis analysis. Rheological analysis of CBG gel revealed that the storage modulus was greater than the loss modulus at all the frequency and suggested the elastic nature of the gel. About 50% weight gain within 12 hours during swelling studies and 50% weight loss within 12 hours during evaporation suggested the suitability of CBG gel as drug carriers. The drug release studies infer, there was an initial burst and later the release was sustained. CBG gel promotes cell migration and demonstrates radical scavenging behavior. When subcutaneously injected into the animal, as *in situ* CBG gel, the gel was highly biocompatible and does not influence any necrosis and the cross talk with the adjacent tissue cells was smooth and the gel completely degraded within 24 days. The wound healing efficacy on full thickness wound suggested that CBG gel accelerate healing and impart high strength to the healed skin at appreciable level. With all these added functional properties, CBG gel finds biomedical applications.

**Keywords**: Gelatin, Bioconjugation, insitu hydrogel, biocompatibility, wound healing

## Introduction

Wound healing is a complex architecture which involves overlapping phases including, inflammation, proliferation and tissue remodelling. Various cellular events and ground substratum matrix were act together to reconstruct injured tissues.[1] In the clinical practice, to enhance the rate of wound healing external agents like scaffolds and hydrogel were employed as wound closure devices, hemostatic agents, tissue sealants and drug carriers.[2-3] Thus, preparation and application studies on hydrogel receive wide attention in the world of biomaterials research. Involvement of biological component in the said preparation considerably increases the functional value (biocompatibility, immune compatability, mechanical stability and controlled degradability) of the material for biomedical applications.[2-3]

In the recent past, numbers of biological materials are in reports[5-9] including hydrogels to satisfy the demand in tissue engineering and drug delivery.[10-13] Hydrogels are three dimensional cross-linked network structures of polymers and has the ability to hold significant amount of water.[14,15] The mechanical strength, biocompatibility and physicochemical similarity to the native extracellular matrix are the added advantages of the high end application of the hydrogels.[16,17] Research on hydrogel initiated during the year 1961[18] to solve the problems associated with contact lenses followed by the introduction of hydrogels from degradable and safe polymers like poly (hydroxyl acids)s, viz., PGA - Poly(glycolic acid); PLA- Poly(Lactic acid); PLGA (Poly(Glycolide-Co-lactide); PCL- Poly-ξ-caprolactone.[19] In addition to biocompatibility issues, gelling mechanism, nature of cross-linkers and cross-linking mechanisms, phase transition, environmental conditions like temperature and pHs have also been considered as the basic requisite parameters when the

application studies on hydrogels are concerned. Furthermore, the mechanical properties and the degradation properties of the hydrogels are also lined up to match the demand.

Injectable hydrogels and scaffolding materials from natural polymers like collagen, gelatin, hyaluronate, fibrin, alginate, agarose and chitosan,[20] the following issues like, purity, endotoxins, immunogenicity, strength, degradation, cellular interactions, cell adhesion and proliferation, porosity, water holding capacity, drug release property, gelation temperature and gelation pHs, were studied and reported by number of researchers.[21-26] It has been understood that most of the hydrogels have less strength, not able to deliver the drug to the targeted site and the homogeneity of the drug loading property is also questionable and the comprehensiveness in hydrogels could not be achieved. Infact, the combinations of two natural polymers impart the desirable strength and stabilization or crosslinking with biocompatible cross-linkers are in reports for the best suitable hydrogels.[21,27,28] Though, the porosity and the water holding capacity of the material determines the application studies,[29,30] the physical nature of the gel decides the method/ mode of application. Recently, instead of preformed hydrogel, *in situ* hydrogels, i.e, injectable hydrogels are in the limelight, where, the material's pattern by itself and able to fit on the wound site. [17,31]

Caffeic acid (3-(3,4-Dihydroxyphenyl)-2-propenoic acid) is a plant phenolic, widely present in vegetables, fruits, coffee, tea, olive oil, red wine etc.[32] This yellow solid consists of di-hydroxyl and α, β unsaturated (acrylic) carboxylic groups. Caffeic acid imparts several functions like wound healing, antimicrobial, antioxidant, anti-inflammatory, antitumor, antianxiety, antimetastatic, anti tumour and inhibits HIV replication. Literatures prove that caffeic acid can able to inhibit several biological enzymes like Matrix Metallo Proteinase (MMP 2 & 9), lipoxygenase, cycloxygenase, glutathione s- transefrase and xanthine oxidase activity etc.[33] Caffeic acid is one of the least toxic compounds amongst various phenolics screened for cytotoxicity studies using fibroblast cell lines.[32] Most importantly, caffeic acid

has similar structural and functional resemblance of DOPA, a potential candidate imparting adhesive strength and curing rate of mussel adhesives.[34]

Gelatin is an age old protein derived from the hydrolysis of the parent protein collagen. On account of its biodegradable, biocompatible nature, lower immunogenicity and its commercial availability at low cost, it has been used since ancient times for food and pharmaceutical purpose.[35] Gelatin can be crosslinked or modified with the inclusion of other materials to significantly alter its mechanical and biochemical properties, used in the preparation of bioadhesives, scaffolds, cell sheet carriers and hydrogels.

In the present study, an attempt was made to have the improved mechanical strength and biocompatible properties of multifunctional injectable hydrogel of denatured protein, which resolves the problems associated with the existing hydrogels of protein origin. In brief, di-hydroxy phenolic acids (Caffeic acid) conjugated with gelatin and the oxidation of phenolics using mild oxidizing agents transforms the conjugated protein to a hydrogel. The hydrogel obtained was then subjected to physico-chemical and mechanical characterization in addition to *in vitro* and *in vivo* assessment of biocompatibility, biodegradability and wound healing properties.

## Materials and methods

### Materials

Gelatin, type A (300 bloom), Caffeic acid (3-(3,4-Dihydroxyphenyl)-2-propenoic acid), Collagenase- type I (*Clostridium histolyticum*), Lidocaine hydrochloride monohydrate and MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)were procured from Sigma Aldrich, USA. Sodium meta-periodate from Sd-fine chemicals, India; Calcein AM, a cell-permeant dye purchased from Invitrogen, India; Ketamine Hydrochloride obtained from Neon Laboratories, Mumbai India; and Fibrin glue obtained from Reliance Life Sciences,

Mumbai, India, in the form of Reliseal. All the other chemicals and reagents used were of analytical grade and commercially available.

**Preparation of Caffeic acid Bioconjugated Gelatin**

Bio-conjugation of caffeic acid to gelatin made using EDC-NHS.[36] In brief caffeic acid dissolved in 0.1 M MES buffer (pH 4.5-6.0) mixed with EDC and NHS at 1 : 2 : 2 mole ratio, stirred for 25-45 min at 25 ºC and then mixed with gelatin dissolved in HEPES buffer (pH 6.5-8.0). The resulting solution was extensively dialyzed against 5 mM HCl and then against the same medium containing 1 % NaCl. Final dialysis was made against 0.1 M phosphate buffer (pH 6.5- 7.5), the samples were then lyophilized and stored at 4 ºC and named as CBG (Caffeic acid Bioconjugated Gelatin). Caffeic acid bioconjugation was confirmed using UV- Visible and $^{1}$H- NMR spectral analysis.

**Preparation of CBG hydrogel**

CBG gel was prepared according to the procedure summarized below. In brief, 2 ml of 10 % CBG solution (10 % of CBG dissolved in phosphate buffer saline) taken in a covered glass container mixed well with 25-50 µl of 1.0 % sodium meta periodate. The time taken by the CBG solution to gel state was measured as curing (gelation) time. The resulting product was named as CBG gel.

**Characterization studies on CBG gel**

Gelatin, CBG and CBG gel samples were subjected to viscosity, contact angle, zeta potential, surface morphology, fluid uptake ability, rate of evaporation and rheological characterization studies as summarized below;

The change in the apparent viscosity of gelatin and CBG was measured using the Brookfield rotational viscometer,[37] spindle No. S18 at 37 ºC at 50 RPM and the concentration was fixed at 6.67 %. Type I water was used for dissolving the samples. In order to measure the change in the surface charge of the protein upon conjugation with caffeic acid, zeta

potential measurements were made using Malvern Instruments, UK.[38] Gelatin and CBG samples were separately dissolved in deionized water and the zeta potential values measured at the default parameters of dielectric constant, refractive index and viscosity of water. Further, the contact angle measurements were made for native gelatin and CBG using Easy Drop, KRUSS, Germany, to assess the spreadability or wettability of the samples[39]. In brief, the samples were cast into a thin film of approximately 0.1 mm thickness. Ten microliters of sterile Type I water was applied on the surface of the film using a 22-gauge needle. The angle measured using the imaging software provided by the supplier. The surface morphology of the CBG gel was assessed using scanning electron microscopy as described below (SEM - HITACHI-S3400N SEM operated at 5kV).[35] The CBG hydrogel sample was freeze-dried and the cross section of the freeze-dried samples was placed on the carbon ribbon and gold coated. The cross sectional view was observed and captured at different magnifications.

**Fluid uptake and rate of evaporation measurements for CBG gel**

The fluid uptake ability of CBG gel was determined according to the procedure summarized.[20] In brief, 2.5 ml of 10 % of CBG dissolved in PBS was taken and oxidized to form a CBG gel. Later, the gel was placed in the closed container containing saline and incubated at 37 ºC for the period of 48 hours. At regular intervals the samples were removed and made free from PBS by gently blotting with a filter paper and measure the weight till attaining the equilibrium. The following formula was used to measure the fluid uptake ability in percentage; equilibrium fluid content (%) = $(W_s - W_i)/ W_i$ X 100, where,$W_s$ and $W_i$ represent the weight of the swollen and initial weight of the sample respectively. The results were plotted based on the average of three individual experiments.

With reference to the rate of evaporation studies, CBG gels prepared as described above were kept at 37 °C and at 35 % relative humidity for the period of 48 hours. 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.

**Rheological studies on CBG gel**

Rheological analysis of CBG gel was performed using an oscillatory rheometer (Anton Paar Rheometer MCR-301) of plate geometry, 8 mm diameter with 0.1 mm gap. The experiments were carried out at 37 ºC using 10 % strain and the frequency varied from 0.1 to 100 rad/s. The change in viscoelasticity was recorded as storage (G′) and loss modulus (G″). Results were obtained as an average of three independent experiments for each concentration of CBG gel studied. Based on the rubber elastic theory, the average mesh size (ξ) was calculated using the following formula[25]

$$\xi = (G'N_A/RT)^{-1/3}$$

The average molecular weight ($M_c$) between the crosslinks were calculated as

$$M_c = (c\rho RT/G'),$$

where G′ is the storage modulus, $N_A$ is Avogadro constant, $R$ is the molar gas constant, $\rho$ is the density of water and $T$ is the temperature (K).

**Radical scavenging behavior of CBG gel**

Gelatin, Caffeic acid, CBG and CBG gel of various concentrations were subjected to assessment on radical scavenging efficacy using $DPPH^{\bullet}$ at room temperature under dark condition.[40] Gelatin, CBG and CBG gel 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 were added. After the scheduled time interval, 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, CBG and CBG gel were taken as control and sample without DPPH were taken as blank. The standard consists of α – tocopherol (10 mg/ml) in absolute alcohol. Based on RSA, $IC_{50}$ values were calculated and compared with the standard. The $IC_{50}$ represents the concentration of the samples responsible for inhibition of 50 % of radical activity of DPPH.

**Antimicrobial profile analysis of CBG gel**

The antimicrobial property of CBG gel was performed according to the methods summarized.[41] Gram-ve and Gram + ve bacterial species were chosen as the test organism. In the lawn of test organism, the preformed gel was placed at the center and incubated for the period of 24 hours at 37 ºC. The zone of inhibition exhibited by the gel was measured and expressed in millimeter. Respective antibiotic discs were also tested for comparison.

***In vitro* release**

The efficacy of the CBG gel to deliver the drug was assessed using lignocaine/lidocaine as a representative drug molecule of interest. The drug was physically mixed with the CBG precursor solution in the concentration of 1.4 mg/ml and the gel was prepared as described above. The gel was then placed in a 20 ml of PBS medium under shaking of 100 rpm at 37 °C. Periodically, 10 µl of the sample was collected and measured the absorbance at 210 nm and the concentration was calculated from the standard graph of lignocaine.

**Biocompatability assessment**

Biocompatibility assessment was made under *in vitro* and *in vivo* conditions. *In vitro* condition is based on the cell adherence and cell proliferation studies as described below.[42] In addition, the cell scratch assay has also been performed to understand the influence of CBG gel on cell migration.

**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), 200 mM Glutamine, 2 mg/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.

**Cell adherence and proliferation**

NIH 3T3 cell adherence and proliferation assessment using CBG gel was made with CBG gel pre-coated culture plates. In brief, 250 µl of 2 % CBG solution was indented to twelve well culture plates and subsequently oxidized with periodate and subjected to air dry at 40 ºC. The control wells were free from the CBG gel coating. The dried plates were then surface sterilized with 70 % alcohol for 30 min and then UV sterilized for 1 hour. The plates were then washed with sterile PBS for 1hr. Cell density of 3 x $10^4$ cells per well was seeded and incubated with the growth medium for the period of 6, 12, 24 and 48 hours and the observations on cell viability, adherence and proliferation made as described below.

The cell adherence and proliferation of fibroblast cells were visualized and quantified by live cell tracker assay and MTT assay methods. With respect to cell tracker assay, after scheduled time intervals (6, 12, 24, 48 hours), the medium was removed and washed with PBS. Calcein AM solution (4 µM; 500 µl) was added and incubated for 30 min. The plates were then washed with PBS and viewed at fluorescence excitation and emission wavelength

of 495/515 nm respectively using a fluorescent microscope with blue filter (Euromex, Holland.). With regard to MTT assay, the culture media of each well was replaced with MTT (5 mg/ml diluted in serum-free medium) and incubated at 37 ºC for 4 hours. Followed by the removal of MTT solution, dimethyl sulphoxide was added and then left at room temperature for two minutes and measured the absorbance at 570 nm using a plate reader (Epoch, BIOTEK).

**Cell migration assay**

Cell migration assay was performed according to the method summarized.[43] NIH 3T3 cells were harvested by trypsinization and loaded in a 48 well plate at a concentration of 5 X $10^4$ cells per well and allowed to form a uniform monolayer. Followed by attaining 80 to 85 % cell confluence, a scratch was introduced using a sterile 200 µl tip. The plates were then washed with PBS to remove the dead cells and then supplemented with DMEM medium containing gelatin, caffeic acid and CBG gel respectively. Wells indented with media alone served as control. Migration of cells and the reduction in empty space measured at 0, 12 and 24 hours of incubation using Image J software.

**Biodegradability assay: *In vitro* assessment**

Biodegradability of all the experimental samples was assessed according to the procedure summarized.[28] Ten milligram of oven dried samples of gelatin, CBG, Glutaraldehyde crosslinked gelatin (Positive control) and CBG 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 hours.

**Biocompatibility and Biodegradability assays : *In vivo* condition**

*In vivo* biocompatibility of injectable CBG gel was assessed by adjacent tissue response analysis in animal models. Necessary approval from the animal ethical committee;

vide no. 466/01a/CPCSEA – IAEC No. 10/2013(a) was sought. Twelve female albino (Wistar strain) rats with an average weight of 200 ± 25 g were used for the study. The animals were housed in standard animal cages and fed with pelletized feed and surplus water. The house temperature was maintained at 25 °C with 12 hours light dark cycle.

At the time of experimentation, animals were anesthetized with ketamine (80 mg/kg body weight) intraperitoneally. The implant area was shaved and sterilized with surgical spirit. *In situ* gel formation under the subcutaneous skin was made by injecting 1.5 ± 0.1 ml volume of CBG in solution form along with the periodate solution. The resulting hydrogel (cured within three minutes) imparts a raised skin appearance. The rats were then allowed to recover and allowed free access to water and feed. Sampling was done at 1, 2, 3, 7, 14, 24 and 36 days. Observations on erythema and edema and skin sensitivity were made. During sampling, the rats were euthanized by cervical dislocation and the pelt was harvested without disturbing the implant and washed with sterile PBS and stored in 10 % buffered formalin for 1 week and later embedded in paraffin wax for sectioning. The samples were cut into half vertically along the skin-implant-fat layers. A 5 μm thickness sections was prepared using a microtome and then stained with hematoxylin and eosin and the images were observed under Nikon eclipse 80i microscope. The sections were analyzed for blood material interaction, provisional matrix formation, acute/chronic inflammation, granulation tissue and fibrous capsule formation.

***In vivo* wound healing studies: Animal model**

*In vivo* wound healing property of CBG gel was assessed by excision wound model followed by approval of the ethical committee. Eighteen female albino (Wistar strain) rats with an average weight of 200 ± 25 g were segregated into three groups (Group I Control; Group II CBGgel treated; Group III Fibrin sealant treated) and six animals for each group.

The animals were housed in standard animal cages and fed with pelletized feed and surplus water. The house temperature was maintained at 25 ºC with 12 hours light dark cycle.

Animals were anesthetized with ketamine (80-100 mg/kg body weight) intraperitoneally. A 4 $cm^2$ area (2 X 2 cm) full thickness excision wound (square shape) was made in the dorsal side of the rat. About one ml of CBG sample was applied to the wound site along with periodate (<50ul) for *in situ* gelation. Similarly, the positive control group receives 1 ml of fibrin sealant. The animals were observed periodically for 4,8,12,16 and 21 days and the post wounding granulation tissue was removed and subjected to quantification of hydroxyproline,[44] hexosomaine[45] and uronic acid[46] content.

The wound margin/area was traced on a transparent paper having a millimeter scale, and the reduction in wound size was measured planimetrically. The period of re-epithelialization was calculated as the number of days required for the wounds to heal completely without any raw wound left behind. The tensile strength of the healed tissues was measured finally. The healed tissue was processed for histopathological studies with hematoxylin and eosin staining.

**Results and Discussion**

The current scenario on diseases and the targeted drug delivery systems necessitates the biobased materials to circumvent the various pathophysiological issues. Hydrogels received high attention due to its unique properties like, water absorption and the structural resemblance to the extracellular matrices. The water holding capacity and the 3D network, facilitate the diffusion of biomolecules. This excellent permeability makes the hydrogels a good transporter of cells, nutrients and drugs. Most of the hydrogels of synthetic and natural origin in reports is targeted to deliver the therapeutic proteins. Further, tailoring and tuning of a backbone molecule of hydrogel has been attempted by a number of researchers to keep

the integrity of therapeutic drugs till it reaches the targeted site and also to hamper the initial burst. It has been suggested by the researchers[17,31] that compared to pre-formed hydrogels, *in situ* gel formation resolves the associated problems and thus, injectable hydrogel came into limelight. But, the major drawback was associated with the precursors employed. Meager changes in precursor concentration exhibit high impact on the gel modulus and its degradability inside the body system. Further, the internal milieu has influence on the delivery of drugs. Assessing the crosstalk between the implanted hydrogel and the tissues around the gel may infer more information on the suitability of the hydrogel for biomedical applications. Moreover, the research inputs on delivery of drugs other than proteins through hydrogel is meager and needs much exploration.

By keeping the above said information, in the present study an attempt was made to impart additional requisite (wound healing, drug delivery, antioxidant property and antimicrobial) properties to the hydrogel by conjugating the di-hydroxy phenolic acids to gelatin followed by oxidation with mild oxidation agents. Gelatin, a denatured protein molecule of collagen origin has a unique property, namely biocompatibility and biodegradability and thus has extensively been exploited in the field of biomedical research.[47] The less stability of gelatin, however, has been managed very well by cross-linking with chemicals such as glutaraldehyde. However, the recent realization on toxicity restricts the use of glutaraldehyde in the biomaterial preparations.[22] Recently, an alternative in the form of dicarboxylic acids as a stable, cross-linker and as a dissolution agent has been reported by the researchers.[48] In the case of injectable hydrogel preparations, attempts were made on teathering the gelatin backbone with molecule of interest, which, ultimately improve the strength and other biological properties. Dongargaonakar *et al*[49] covalently conjugate highly branched dendrimer Polyamidoamine to gelatin via EDC/NHS for wound dressing and drug delivery. Similarly, in the present study, dihydroxy phenolic acid conjugated gelatin was

prepared using EDC/NHS. The resulting caffeic acid conjugated gelatin (CBG) has been used as a source material for the study. UV-visible spectrum of the CBG showed peaks at 290 and 315 nm confirm the presence of caffeic acid in the gelatin backbone. Similarly, the chemical shift values at δ 6.3, 6.9 and 7.1 ppm obtained from the $^{1}H$ NMR spectra (Supplementary file) confirms the effective bioconjugation of caffeic acid to the gelatin.[36]

Caffeic acid bioconjugated gelatin (CBG), when exposed to periodate the transformation from solution state to gel state occurs, which depends on the concentration of CBG. The chemical reaction involved in the gel formation may be explained based on the chemical structure of caffeic acid, which reveals that the presence of adjacent hydroxyl groups undergoes oxidation and formation of electrophilic molecules such as quinones. The intermediate semi-quinone radicals and the phenolic anions readily undergo nucleophilic addition reaction with the protein side chains (thiols of cystiene and methionine, amines of lysine, N-terminal polypeptide, asparagines and glutamine, hydrophobic aromatic group of tryptophan and tyrosine of proteins) results with the inter and intra molecular crosslinking facilitated through (i) Michael's addition reaction (ii) Schiff base formation and (iii) biaryl coupling reaction and transform to a supra macromolecular structure called hydrogel (Fig. 1).[34,50-52] Similarly, Choi *et al*[52] highlights the possibility of transformation of tyrosine in the gelatin backbone to DOPA, which upon complex with $FeCl_3$ transformed to a hydrogel. It seems that the percentage availability of tyrosine molecule (10 residues/1000 amino acid residues) decides the gelling property and which was considerably less compared to the present study, in which, the target molecule is primary $–NH_2$ group (lysine, 28 residues/1000 residues), which upon bioconjugation and oxidation impart expected intermolecular interactions results with appreciable gel strength. It has been understood that all the said reactions depend on pH, temperature and oxygen availability and in the present study, the oxygen availability has been taken care by the $IO^{4-}$. The nucleophilic substitution

reaction further reduces the quinones to dihydroxy phenols, which in turn undergoes oxidation and cross link with another polypeptide chain, resulting in the formation of *in situ* gel as shown in Fig.1.

Recent studies revealed that modification of natural polymers with bioactive epitopes improved the biological property of a hydrogel at a considerable level[53]. Similarly, Kyung Min Park reported *in situ* cross-linkable gelatin–poly(ethylene glycol)–tyramine hydrogel via enzyme-mediated reaction for tissue regenerative medicine[54].

**Physical characterization**

With regard to physical characterization of CBG hydrogel (Table 1), viscosity is one of the major criteria for an injectable hydrogel and it has been suggested that for homogeneous dispersion of drugs, gels with low viscosity before gelation is highly preferable.[17] Viscosity measurements for CBG gel made at 37 °C suggested that there was 29 % increase in viscosity compared to the parent protein gelatin. Viscosity of gelatin before conjugation was 8.3 cP, and for CBG was 11.7 cP after conjugation. The increase in the viscosity of CBG could be reasoned to the increase in molecular weight of gelatin upon conjugation. The ortho and para directing hydroxyl group of caffeic acid is involved in the hydrogen bonding interaction with the -NH and -CO group of gelatin thereby increasing the molecular interaction. The addition of caffeic acid to the gelatin backbone reduces the availability of primary $-NH_2$ group and appropriately decreases the hydrophilicity. The presence of alkyl chain and the aromaticity in the caffeic acid further increases the hydrophobicity of the protein, which is reflected in the viscosity.

Zeta potential measurements made with gelatin and CBG revealed that upon conjugation the charge value of gelatin decreased to 28 %. The actual values measured for gelatin and CBG were 4.7 and 3.67 meV respectively. The reduction in charge value for CBG could be due to functionalization with caffeic acid. In general, predominantly the

positive charge of the proteins is due to its the primary -$NH_2$ group. During bioconjugation the interaction of -$NH_2$ groups with caffeic acid resulted with the reduced charge. Moreover, in the present study, the measurements were done in the presence of water as solvent. The pH of the solution was around 7, which is the isoelectric point of Type A gelatin (pI 7.0 – 9.0) which reflects on the zeta potential measurements, which is near to zero. Sushma Kommareddy[38] also observed a decrease in charge value of gelatin to near neutral when conjugated with thiols.

Contact angle measurements on thin film of gelatin and CBG samples displayed theta value of 66.2° ± 0.8 and 82.7° ± 1.2 as the angle of wettability respectively. The significant increase in the contact angle of conjugated gelatin compared to gelatin could be due to the presence of alkyl chain and the aromaticity of the caffeic acid. The observed contact angle values less than 90° implied that good wettability of the surfaces and add value to the conjugated protein for its interaction with tissue surfaces when indented to the body system under live condition. Similarly, Xi Yang *et al*[55] found an increase in contact angle of dopamine –coupled human gelatin suggesting the reduction in the hydophilicity. Scanning electron microscopic images of CBG gel revealed the presence of a well defined three-dimensional matrix with porous structure attributed to the intra and intermolecular cross linking (Fig.1). The porous structure facilitates the water absorption and promotes cell adherence, proliferation and molecular diffusion as evidenced from the *in vitro* and *in vivo* studies.

**Fluid uptake ability**

The fluid uptake ability is the important parameter of a hydrogel when subjected to wound healing applications, in which, the excess wound exudates/fluids absorbed by the gel facilitate the healing process extensively. In the present study, fluid uptake ability of CBG gel was assessed using physiological saline at 37 °C for the period of 48 hours. The increase

in weight measured at different time intervals suggested that within 8 hours of incubation, the gel gains 38 % extra weight and after 12 hours the weight gain was 44 % and attained equilibrium as shown in Fig. 2a. It has been understood that the inter and intermolecular crosslinking of the CBG does not allow the gel to swell more than 50 % of its initial weight, which is an impressive positive factor where, the mechanical properties of the gel will be maintained.

Similar to the fluid uptake, rate of evaporation is also a significant parameter for a hydrogel. The loss in weight upon 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.[21] In the present study, CBG gel losses its 50 % weight over the period of 12 hours and after 24 hours no significant weight loss was observed (Fig. 2a). Results on the fluid uptake and evaporation loss studies, it has been understood that CBG gel capable of absorbing the fluid to the level of ~50 % of its original weight and release the same and reach the original shape within 12 hours. If we consider the wound surface, at the time of application, CBG gel may absorb the wound exudates and at the same time maintain the moist environment, which promotes granulation, re-epithelialization, and autolytic debridement. According to Balakrishnan et.al[21] moist environment makes the wound cool and reduces the pain. Further, the authors observed, application of hydrogel reduces the period of dressings at considerable level. Commercially available hydrogel, Geliperms (Geistlich Ltd., Switzerland) shown similar behavior (fluid uptake and evaporation loss) like CBG gel, by retaining 30 % of water after 24 hours. According to Ganji *et al*[56] on theoretical studies on swelling suggested that the size of pores determines the equilibrium swelling and the super porous hydrogels (SPHs), 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 mater of a minute regardless of their size. In the present study,

equilibrium state was obtained within 8 hours, suggesting the microporous structure of CBG gel.

**Rheological characterization**

The rheological profile of CBG gel with reference to the concentration of CBG illustrated in Fig. 2b. It has been observed that gelatin upon conjugation with caffeic acid displayed less storage modulus (G′) than the loss modulus (G″) when examined at lower frequencies. However, at higher frequencies, the reverse was observed, which indicate at lower frequencies conjugated gelatin behave as a viscous liquid, and as the frequency increases it behaves as a gel (not shown). With respect to CBG gel, irrespective of the frequencies, the storage modulus was greater than the loss modulus, implied that CBG undergoes uniform cross linked network. When increasing the concentration of CBG gel from 8 to 12 %, a significant increase in storage modulus was observed. The observed storage modulus of CBG gel was comparatively higher (1100 Pa) than the hydrogel obtained from DOPA modified gelatin (10 Pa) cross-linking with tyrosinase and $Fe^{3+}$. Young Chan Choi,[52] reported that the rheological property of gelatin based hydrogels can be controlled by the degree of polymer concentration and degree of substitution. The mechanical spectra revealed that the CBG gel behaves as an elastic solid rather than a viscous liquid. The tan δ value for the CBG gel (8 to 12 %) decreases from 0.154 to 0.043 (Table 2), suggested that the elastic nature of the material increases with the increasing crosslinking density. However, CBG gel prepared at 10 and 12 % concentration does not show any significant variation. This could be attributed that increase in the concentration of CBG increases the steric hindrance of the protein chain which disturbs the mobility of the protein undergoing crosslinking. From the elastic property of the CBG gel, we can able to establish the relationship between the

CBG concentration, the network structures and theoretical mesh size according to Affine network model.[25] The mesh size and the average molecular weight between the crosslink was calculated based on the peak storage and loss modulus of the CBG gel. When the concentration of the CBG increases from 8 to 12 %, the mesh size decreased from 29 to 13.9 nm, which implies, when the crosslinking density and the protein concentration increases the porosity decreases which correspondingly decreases the average molecular weight between the crosslink as shown in Table 2. Further, it has been suggested that the mesh size of hydrogel should be high compared to the hydrodynamic radii of the drug or the cell for free mobility or diffusion.

***In vitro* drug release studies**

In order to assess the drug release properties of CBG gel, Lignocaine, a antiarrhythmic drug (local anesthetic drug for minor surgeries) was chosen as a model drug. Fig. 2c depicts the drug release profile of the CBG gel. Observations on 20 % of the drug release within 4 hours could be reasoned to the surface bound drug. Whereas, release of 50 % drug was realized around 15 hours and more than 90 % release was observed at 48 hours of incubation. Since the drug release pattern of hydrogel depends on the swelling ratio, which in turn differs with the pH of the medium. In the present study, it has been observed that at pH 7.4 CBG gel gains 50 % of its weight and at pH 1.0 and 3.0 the weight increase was calculated as 300 and 500 % (Supplementary file). As discussed, the hydrodynamic radii of the drug play an important role in the release and if it is less than mesh size of the hydrogel it will be preferable for the delivery. In the present study, the hydrodynamic radii of the drug Lignocaine was 0.5 nm and the mesh size of the CBG gel calculated from the rheological studies was 15-30 nm suggested the possible diffusion of the drug. Further, the swelling ratio and the release profile correlate well with CBG gel. The controlled release pattern of

drug by CBG hydrogel at physiological pH observed in the present study suggested the suitability of CBG gel for sustained release of drugs.

**Antioxidant property**

It is well known that wound milieu contains various inflammatory signals and reactive oxygen species (ROS). These ROS attack the healthy tissues surrounding the wound and slow down the healing process. If these radicals are scavenged properly, the healing may be accelerated at a greater level. In general, most of the hydrogels are designed to absorb the wound fluid and also to deliver the therapeutic drugs. If the hydrogel is integrated with antioxidant property, it adds value to the functional property of the hydrogel. In the present study, bioconjugation with caffeic acid imparts the antioxidant property to the CBG. Caffeic acid is well known for its antioxidant and anti-inflammatory effects and it also inhibits the activity of proteolytic enzymes at a considerable level.[57] In the present study, the antioxidant activity of CBG and CBG gel at different concentration was tested for their ability to scavenge DPPH free radical. A 50 % reduction in the free radical activity of DPPH was observed for 175 and 350 µg concentrations, respectively to CBG and CBG gel. the standard tocopherol displayed 50 % reduction at 2 µg concentration (Fig. 2d). The presence of proton donating residues in the form of hydroxyl groups and the unsaturation in the bioconjugated caffeic acid is responsible for the antioxidant activity of the gel. According to the available reports, aromatic hydroxyl groups are more responsible for the antioxidant activity of the samples. Sonia Trombino *et al* incorporated the antioxidant activity to the cellulose based hydrogel using trans-ferulicacid.[58] 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 hydroxyl groups.[59]

**Antimicrobial profile of CBG gel**

Antimicrobial assessment of CBG gel with selective Gram +ve and Gram-ve bacterial species suggested that the zone inhibition of 17 ± 0.8 mm was observed with Gram-ve bacteria (*E.coli*) and only less than 2 mm with Gram +ve bacteria (*B. subtilis*) (Supplementary file). Further, we found no growth on the surface of the gel and no recurrence. The antimicrobial property exhibited by the CBG gel could be reasoned to caffeic acid and iodate ions.

**Cell adhesion and proliferation profile of CBG gel**

The property of cell adherence and proliferation of a biomaterial is essential when *in vivo* application studies are concerned. In the present study, cell adherence and cell proliferation potential of CBG gel was assessed using NIH 3T3 cells. Fig. 3a illustrates the fluorescence image of the cells observed at different time intervals. When cells were stained with calcein fluorescent probe, a cell-permeant dye, in live cells the nonfluorescent calcein AM converted to a green-fluorescent calcein after acetoxymethyl ester hydrolysis by intracellular esterases. In the present study, no significant difference in fluorescence intensity in the CBG gel treated cells compared to the control. Furthermore, no morphological changes was observed in the CBG gel treated cells. Results on cell viability and quantification suggested that more than 90 % cells were viable and bioconjugation of caffeic acid with gelatin does not have any negative impact on cell viability (Fig. 3b). Several researchers reported the effective reduction of levels of apoptosis of neural stem cells and increased cellular viability in the presence of phenolic acids.[32,60,61]

***In vitro* cell migration assay**

Results on cell migration studies of CBG gel using NIH 3T3 cells suggested that compared to control (untreated) and gelatin alone, caffeic acid and CBG gel treated cells migrate at the fastest rate and more than 90 % of scratch area was covered by the cells within 24 hours (Fig. 3c). The observations suggested that CBG gel influences the cell migration

and add value to the material for its application in wound healing studies. According to Hong *et al* [62] an improved cell migratory effects evidenced in the present study could be reasoned to the free radical scavenging activity of Caffeic acid as evidenced in the present study under antioxidant property.

***In vitro* biodegradation**

Since, wound area is rich with proteolytic enzymes the degradation of a biomaterial is unavoidable and thus it has been considered as a major drawback and challenging criteria in the biomaterial preparation and applications. Most of the protein based batteries degrade at a faster rate before the wound gets healed and the requirement on biomaterial with controlled degradation property is highly essential. Fig. 3d depicts the biodegradation profile of the CBG gel in comparison with gelatin (control), glutaraldehyde cross-linked gelatin, (both were in the form of thin sheet) and CBG. Gelatin and CBG shows a faster degradation compared to glutaraldehyde stabilized gelatin and CBG gel. More than 40 ± 8 μg of amino acid was released from native gelatin within 12 hours exposure to enzymes and the same quantity was observed after 24 hours with CBG alone and after 36 hours in the case of glutaraldehyde stabilized gelatin. However, CBG gel displayed a slow release of aminoacids and the quantity of amino acid released was less than 20 ± 6 μg even after 48 hours. The slow release of amino acids from CBG gel in the presence of collagenase enzyme suggested the enzymatic stability of the material and this property is an added advantage of CBG gel. The interpenetrating networks due to intermolecular interaction in the CBG gel protect the enzymatic degradation of gel. Further, the proteolytic inhibitory activity of caffeic acid also has been identified for the slow degradation of CBG gel.[57] The controlled enzymatic degradation of CBG gel emphasize the stability of the material which is complementary to

the mechanical stability of CBG gel. According to Lutolff *et al* the controlled swelling and degradation of hydrogels have potential application as drug depot for drug delivery, encapsulating therapeutic proteins and growth factors.[63]

***In vivo* biodegradation and biocompatibility**

With reference to degradation of CBG gel under *in vivo* conditions, in the present study, subcutaneously implanted CBG gel was periodically assessed for its morphology, reduction in shape, and adjacent tissue response at different time intervals. Interestingly, the implants ensured nil influence on erythema or edema (Table 3) formation (Fig. 4a(i), day 0 to day 24) in the skin of the animals till the experimental period. Fig. 4a(ii) represents the schematic information on the injectable CBG gel and its size reduction with respect to experimental period for better understanding. The skin prick test also demonstrates that the subcutaneous implant does not show any loss in the sense of the skin. Further, the macroscopic and microscopic observations of the CBG gel showed a slow degradation and complete dissolution within 24 days (Fig. 4a (i & iii).

H&E staining of the sections of the samples (skin-implat-fat layers) after day 1 of implantation, showed a clear provisional matrix formation on the periphery of CBG gel (Fig. 4b, day1). The provisional matrix formation provides the basement structure and further initiates several biochemical factors, which mediates cellular responses for inflammation, neovascularization and infiltration of inflammatory cells on day 2 observed (Fig. 4b, day 2). Moreover, we found relatively a large number of inflammatory cells at the interface of the implant suggested the active biodegradation process[64]. On day 3, despite the recruitment of more numbers of inflammatory cells in the surrounding tissue there was no hemorrhage or necrosis. As a function of time, the inflammatory cells adhere on the periphery of the hydrogels and infiltrates deep into the implant. The release of proteolytic enzymes by the inflammatory cells degrade the CBG gel matrix resulting with the reduction in the size of the

gel when the experimental period increases. On day 14, more than 50 % of the material degraded (Fig. 4b, day 14) and the remodeling stage begins with the reduction in the blood vessel formation and occasional macrophages. Interestingly, we observed no fibrous capsule (fibrous tissue) around the implant and rather no chronic inflammation till the completion of the experimental period suggested the mass transfer between the implants and the surrounding tissues and maintains the implant function.[65,66] According to Horbett *et al*, absorption of non-specific proteins like albumin or immunoglobulin may prevent the *in vivo* degradation of the implanted materials.[67] However, on day 24, a complete dissolution of the CBG gel implant was observed and the basement membrane matrix was restored without any alterations and suggested the high biocompatible and biodegrdable nature of CBG gel. The high interaction of CBG gel with host tissue as evidenced implied the suitability of CBG gel for tissue engineering application as well as tissue regeneration.[65]

***In vivo* wound healing assay**

Followed by *invivo* degradation studies on CBG gel in rat model, an *in vivo* wound healing study was also done to have more therapeutic input on the biocompatible and biodegradable CBG gel. It is well known that wound healing is a complex process which involves inflammation, proliferation and remodeling stages. The wound milieu, size, location and type are the factors that determine the rate of wound healing. A therapeutic agent selected for the treatment of wounds should ideally improve one or more phases of healing without producing deleterious side effects. In the present study, efficacy of CBG gel on wound healing was assessed in a full thickness (4 $cm^2$) square wound. The wound was treated with CBG gel and fibrin sealant act as a standard for positive control experiments. The rate of wound healing was compared with untreated wound. Fig. 5a depicts the wound healing profile, which revealed that the CBG gel treated group needs only 14 -16 days for complete re-epithelialization, whereas, the fibrin and the untreated groups took 21 days, respectively.

Wound contraction analysis demonstrats prior contraction with CBG gel treatment compared to fibrin and control groups. Fig. 5b illustrates the reduction in wound area with reference to the experimental groups which clearly showed influence of CBG gel on healing of wound. The tensile strength of the healed skin showed a wide variations as 910 ± 15.47 (Pa), 1390 ± 27.8(Pa), 1050 ± 21(Pa) respective to untreated, CBG gel and fibrin sealant treated wound respectively. Similar to the observations on the excised wound upon treatment with the CBG gel, histological examination of healed tissue sections also demonstrated that the healing was faster than compared to the fibrin and untreated group. Significant reduction in the wound area was observed with CBG gel treated wound compared to fibrin sealant and untreated wound. On day 16 more than 50 % of the tissue structures were restored. For CBG gel treated wound, hair follicles were observed surrounding the wound area. When compared to untreated and Fibrin sealant, the number of inflammatory cells is less for the CBG gel treated wound (Fig. 5c).

As evidenced in the *in vitro* studies on equivalent level of cell proliferation (compared to control) and increased level of cell migration in the presence of CBG gel, *in vivo* studies also demonstrated an accelerated centripeatal movement of fibroblasts[68,69] in the CBG gel treated group compared to other two experimental groups. Table 4 depicts the biochemical profile of granulation tissue with reference to healing period. An increase in hydroxyl proline content of the granulation tissue samples of CBG gel treated groups compared to other treatments correlates well with the increased tensile strength of the healed skin. The healing process depends, to a large extent, on the regulated biosynthesis and deposition of new collagens and their subsequent maturation. Restoration of the basement membrane is an essential event during wound healing, as it appears to have a profound role in organization of cells to functional units. During granulation tissue formation, as contraction proceeds and resistance increases, fibroblasts differentiated into myofibroblasts. The presence of

myofibroblasts is considered to be characteristic of tissue undergoing contraction. Apparently greater number of myofibroblasts in CBG gel treated group may be partially responsible for the early wound contraction.

Similarly, the hexosamine estimation also confirms an increase and earlier deposition of hexosmamine compared to the standard and untreated groups. The uronic acid content of the CBG gel treated wound shows an earlier increase on day 4 and started decreases and restores to the standard level. However, fibrin sealant treated group and untreated wound showed an increase in the uronic acid content on day 4 and 8 and later restore to the normal level. Hexosamine and uronic acid are the matrix molecules, which act as the ground substratum for the synthesis of new extra cellular matrix. Uronic acid in the wound attracts fibroblasts and stimulates collagen synthesis by providing more fluid which facilitates greater cell mobility, early remodeling and assists the wounds to heal faster without scar formation. The early increase in hexosamine and uronic acid showed that the fibroblasts actively synthesize the ground substratum on which collagen is laid down. It is reported that there is an increase in the levels of these components during the early stages of wound healing, following which normal levels are restored.[70] From the results, it has been evidenced the suitability of CBG gel for biomedical application.

**Conclusion**

As described, in the present study, caffeic acid conjugated gelatin (CBG) hydrogel was prepared and assessed for its various functional properties under *in vitro* and *in vivo* conditions. Bioconjugation with caffeic acid impart and also improve the rheology, mechanical stability, antimicrobial and antioxidant properties of gelatin. The required mesh size of the diffusion or mobility of drug molecule could also be achieved. The average mesh size of 20 nm of CBG gel suggested that it can able to accommodate and also deliver the

drugs with the hydrodynamic radii in the range of 1- 10 nm. And suggested the suitability of CBG gel to deliver drugs other than proteins. Further, the 50 % gain in weight under physiological pH and a corresponding loss during evaporation implied that CBG gel has appreciable mechanical stability. Furthermore, the slow degradation in the presence of proteolytic enzymes also corroborates with the mechanical stability. A normal cell proliferation and increased cell migration observed under *in vitro* studies on CBG gel agrees well with the *in vivo* wound healing studies and early healing of wounds in the presence of CBG gel. Most importantly, the observations made under *in vivo* host response studies of CBG gel when given as an injectable gel implied nil fibrous capsule formation. All the summarized results suggested that bioconjugation with caffeic acid importantly improved the required functional properties (biocompatibility, controlled biodegradability, antioxidant, antimicrobial and wound healing) of the injectable hydrogel and suitable for clinical applications.

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**Figure Captions**

Fig.1 Schematic representation on bioconjugation of caffeic acid with gelatin and the intermolecular interactions and the plausible mechanism behind the formation of CBG gel under post oxidation

Fig. 2

(a) Rate of evaporation and fluid uptake ability of CBG gel assessed at physiological pH at room temperature.

(b) Rheological spectra of CBG gel at various concentrations and assessment of storage and loss modulus

(c) Sustained release profile of lignocaine incorporated in the CBG gel assessed under physiological condition at room temperature

(d) Comparison of radical scavenging profile of gelatin, CBG and CBG gel assessed using DPPH free radical

Fig. 3

(a) *In vitro* fluorescence images of NIH 3T3 fibroblast cells on adherence and proliferation on CBG gel in comparison with control at various time intervals of 6, 12, 24 and 48 hours. Blue filter was used for fluorescence excitation and emission. The scale bar measures 10 μm

(b) Cell viability assessment based on MTT quantification of 3T3 cell population upon exposed to CBG gel in comparison with control (All the values are the mean ± SD of triplicates) under *in vitro* condition

(c) Influence of CBG gel on migration of fibroblast cells (NIH 3T3) assessed from scratch assay (*In vitro* condition). Image J analysis was used to measure the space covered by the migrated cells at different incubation periods, 0, 12 and 24 hours. The scale bar measures 10 μm

(d) *In vitro* proteolytic degradation of CBG gel in the presence of proteolytic enzymes at different incubation period on comparison with gelatin and 0.1 % glutaraldehyde crosslinked gelatin

Fig. 4

(a) *In vivo* biodegradation and host tissue response assessment of CBG gel.

(i) Represents the macroscopic observations made with respect to days. CBG gel was subcutaneously injected in the form of *in situ* injectable gel;

(ii) the schematic model represents the reduction in size of the injectable CBG gel in the subcutaneous junction of the skin. The yellow colour represents the injected CBG gel

(iii) H&E staining of skin-hydrogel-fat layer showing the reduction (marked as yellow coloured arrow mark) as well as provision matrix formation and degradation with respect to the experimental period in days. The scale bar measures 1 mm

(b) Histopathology and H & E staining of the sections (at 10 and 40 X magnifications) containing skin- implant- fat layers of samples received at 1,2,3,7, 14 and 24 days of experimental period (The solid yellow colour arrow heads indicates the presence of fibroblast cells. The black arrows represent the formation of new blood vessels). The scale bar measures 1 mm (4 x) and 10 μm ( 40 X)

Fig.5

(a) Comparative assessment on healing of full thickness square wound (4 $cm^2$) with reference to control (untreated), CBG gel treated and fibrin sealant treated groups on different experimental days 0, 4, 8, 12, 16 and 21 in albino rats (n=6).

(b) A bar diagram on reduction in wound area ($mm^2$) with respect to experimental groups; Control, CBG gel treated and fibrin sealant treated on different days of the experimental period, (*$P < 0.001$, n=6)

(c) H&E staining of sections of healed skin of experimental groups; Control(untreated), CBG gel treated and Fibrin sealant treated groups at 10 and 40 X magnifications. The solid yellow colour arrow heads marked in the 40 X magnification represent the density of inflammatory, fibroblast and myofibrobalst cells. The scale bar measures 1 mm (4 X) and 10 µm (40 X)

**Table 1 :** Physical properties of gelatin and caffeic acid bioconjugated gelatin (CBG)

| Sample | Viscosity (cP) | Surface charge (meV) | Contact angle (θ °) |
|---|---|---|---|
| **Gelatin** | 8.31 ± 0.3 | 4.79 ± 0.04 | 66.25 ± 0.8 |
| **CBG** | 11.73 ± 0.2 | 3.67 ± 0.03 | 82.79 ± 1.2 |

**Table 2 :** The average mesh size, average molecular weight between the crosslinks and viscoelastic property of CBG gel based on rheological data.

| CBG gel (% w/v) | G′ (Pa) | G″ (Pa) | tanδ G″/G′ | ξ (nm) | $M_c$ (kg/mol) |
|---|---|---|---|---|---|
| 8 | 168 | 25.9 | 0.154 | 29 | 1152.6 |
| 10 | 1400 | 65.7 | 0.046 | 14.3 | 172.89 |
| 12 | 1510 | 65.3 | 0.043 | 13.9 | 192.36 |

**Table 3:** *In vivo* host tissue response analysis of CBG gel on different experimental period.

| | Day1 | Day 3 | Day 7 | Day 14 | Day 24 |
|---|---|---|---|---|---|
| **Erythema** | N | N | N | N | N |
| **Edema** | N | N | N | N | N |
| **Skin sensation** | H | H | H | H | H |
| **Polymorphonuclear cells** | N | N | N | N | N |
| **Macrophages** | N | L | L | N | N |
| **Lymphocytes** | L | L | L | L | L |
| **Necrosis** | N | N | N | N | N |
| **Vascularization** | N | L | L | L | N |
| **Fibroblast** | L | L | M | L | N |
| **Capsule Formation** | N | N | N | N | N |

*****Scores are given based on the physical observation and histopathological analysis.
(H-high, M-Medium, L-Low and N-Nil/Non occurrence

**Table 4 :** Biochemical profile of granulation tissue obtained from the skin excised wound of different experimental groups on different experimental period.

| Biochemical Analysis of granulation tissue | Experimental Period (Days) | Untreated | CBG gel Treated | Fibrin sealant Treated |
|---|---|---|---|---|
| **Hydroxyproline*** **(µg/5mg tissue)** | **4** | 23.0 ± 1.1 | 39.5 ± 3.7 | 30.2 ± 4.5 |
| | **8** | 74.7 ± 1.1 | 110 ± 3.3 | 99.1 ± 4.3 |
| | **12** | 99.1 ± 2.1 | 145 ± 6.9 | 133.7 ± 9.3 |
| **Hexosamine*** **(µg/5mg tissue)** | **4** | 30.9 ± 1.5 | 77.3 ± 2.3 | 56.3 ± 3.9 |
| | **8** | 44.9 ± 2.2 | 40.1 ± 1.2 | 53.1 ± 2.2 |
| | **12** | 21.6 ± 1.0 | 15.9 ± 0.4 | 22.1 ± 1.5 |
| **Uronic acid**** **(µg/5mg tissue)** | **4** | 6.1 ± 0.3 | 7.67 ± 0.5 | 7.2 ± 0.2 |
| | **8** | 6.7 ± 0.3 | 7.1 ± 0.4 | 7.1 ± 0.2 |
| | **12** | 6.4 ± 0.3 | 6.2 ± 0.2 | 6.0 ± 0.1 |

CBG gel treated wound compared with untreated wound *$P < 0.03$ and **$P < 0.1$ (n = 3).

Fig. 1

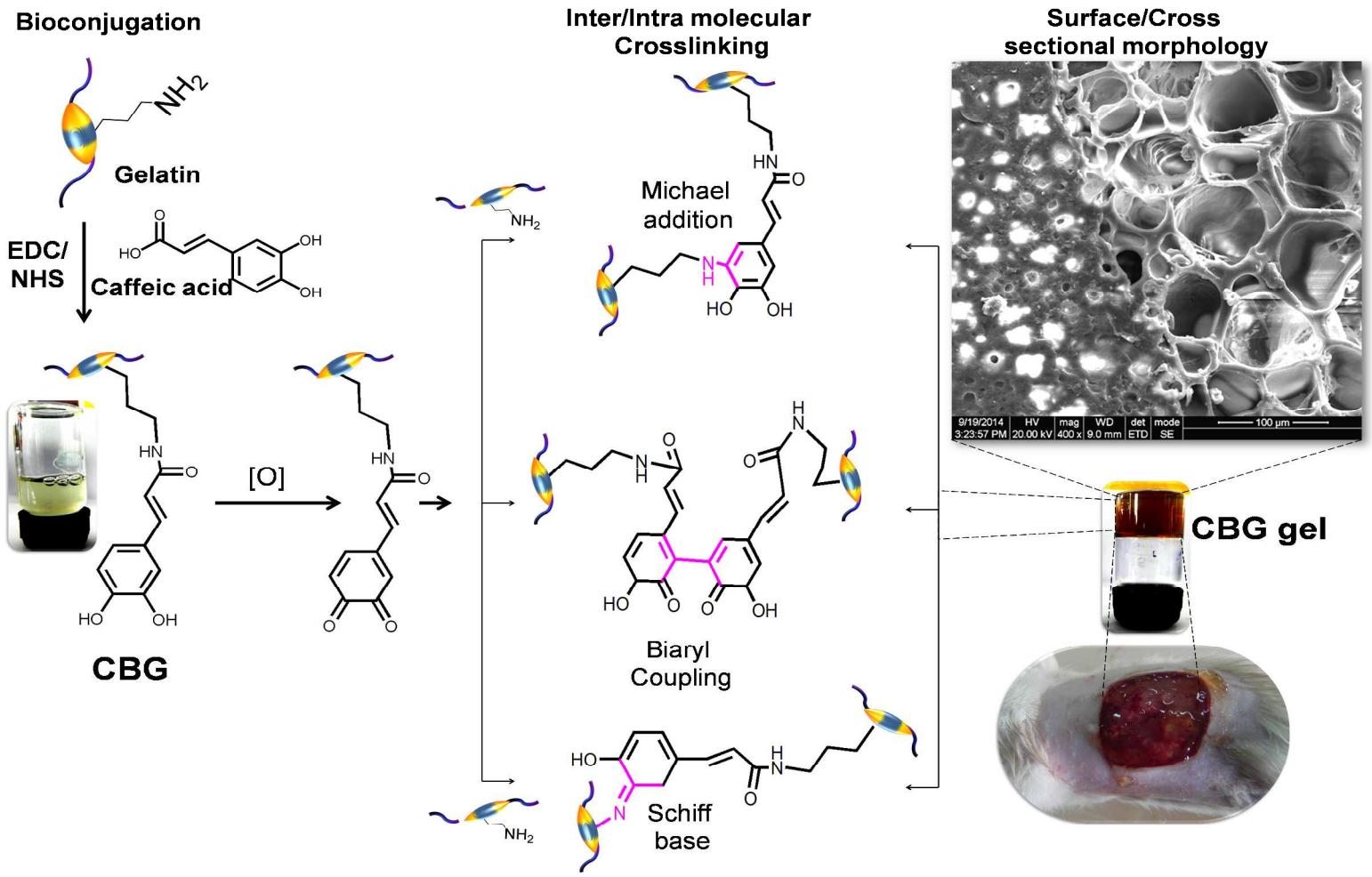


**Fig. 2**

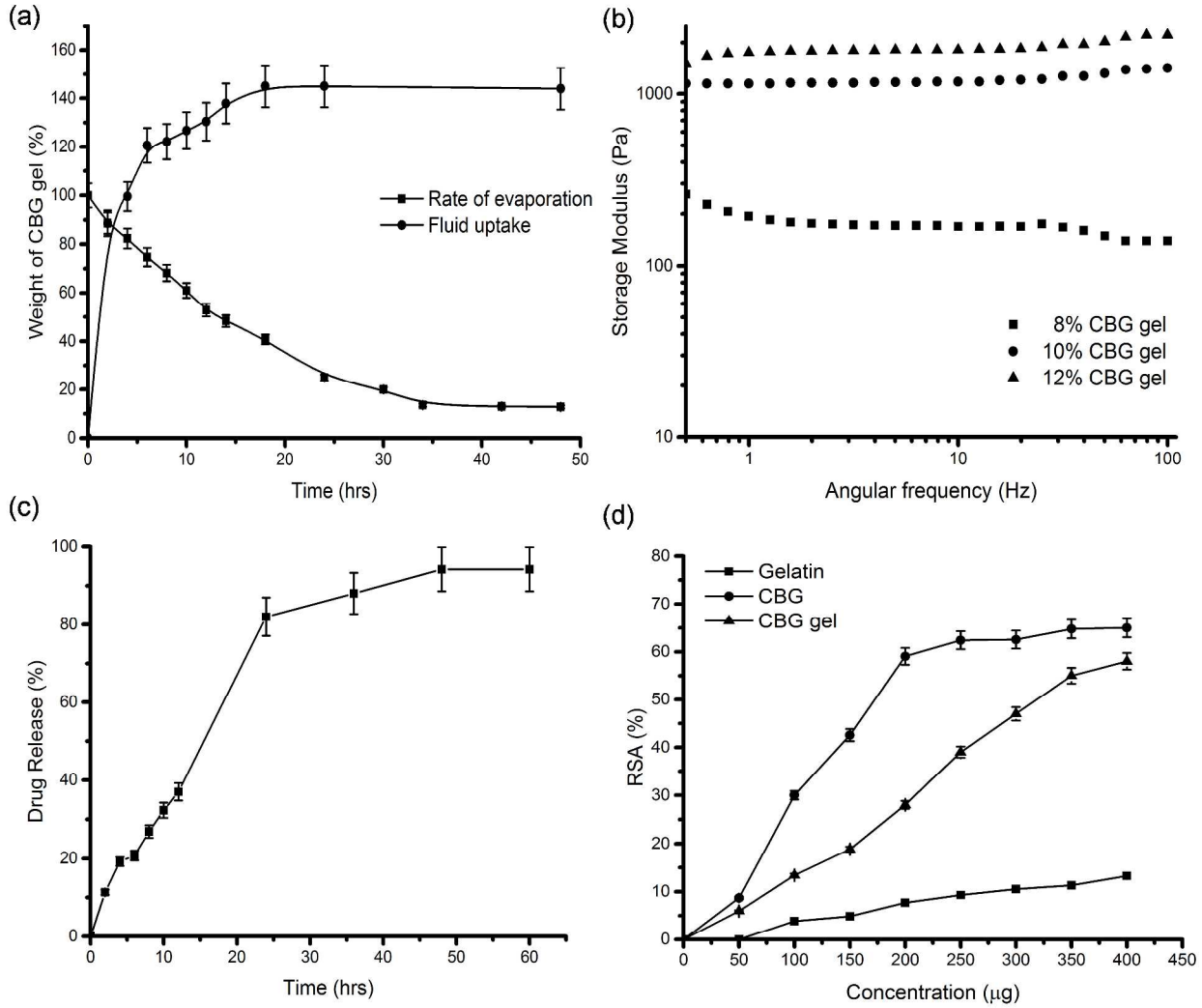
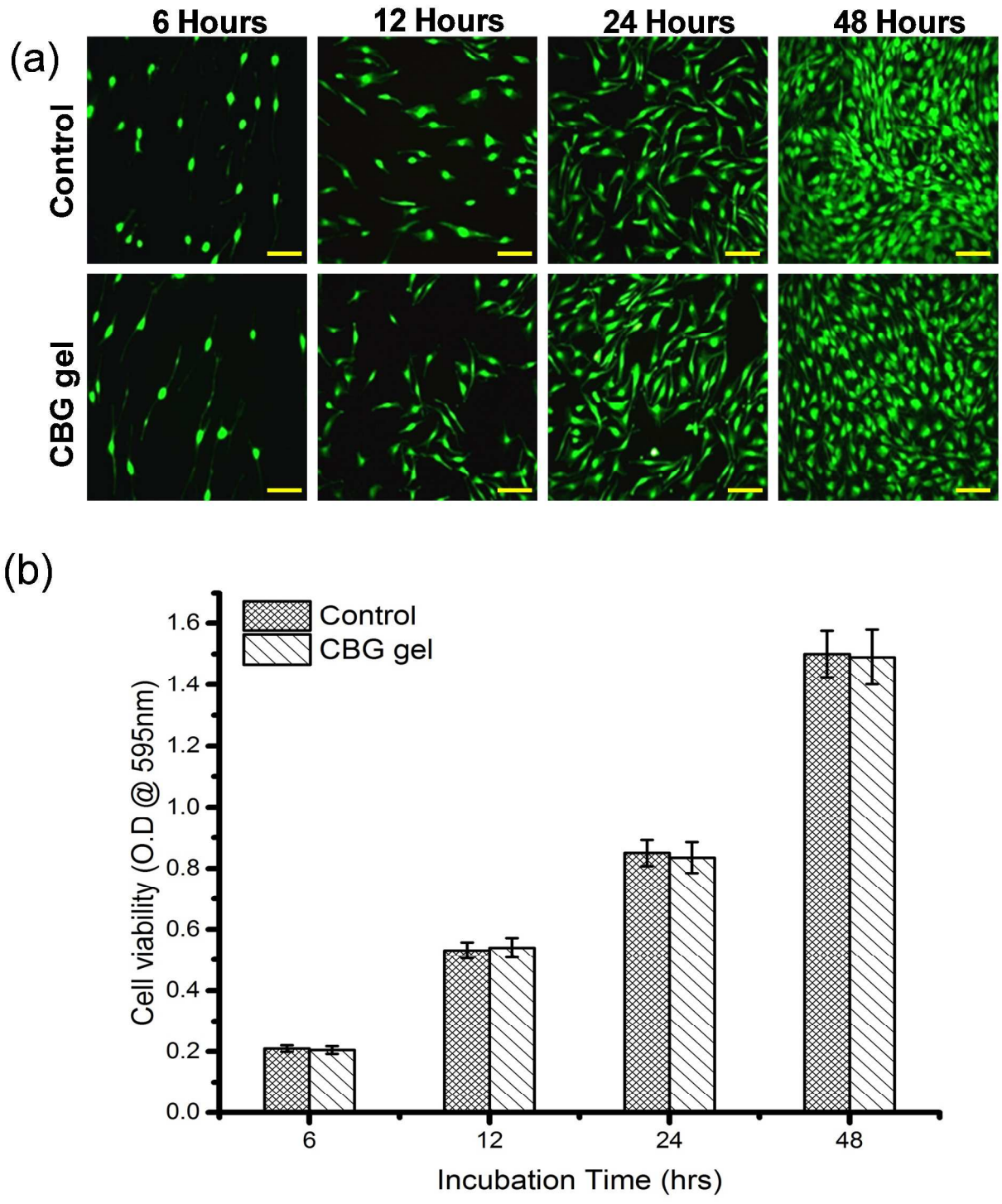
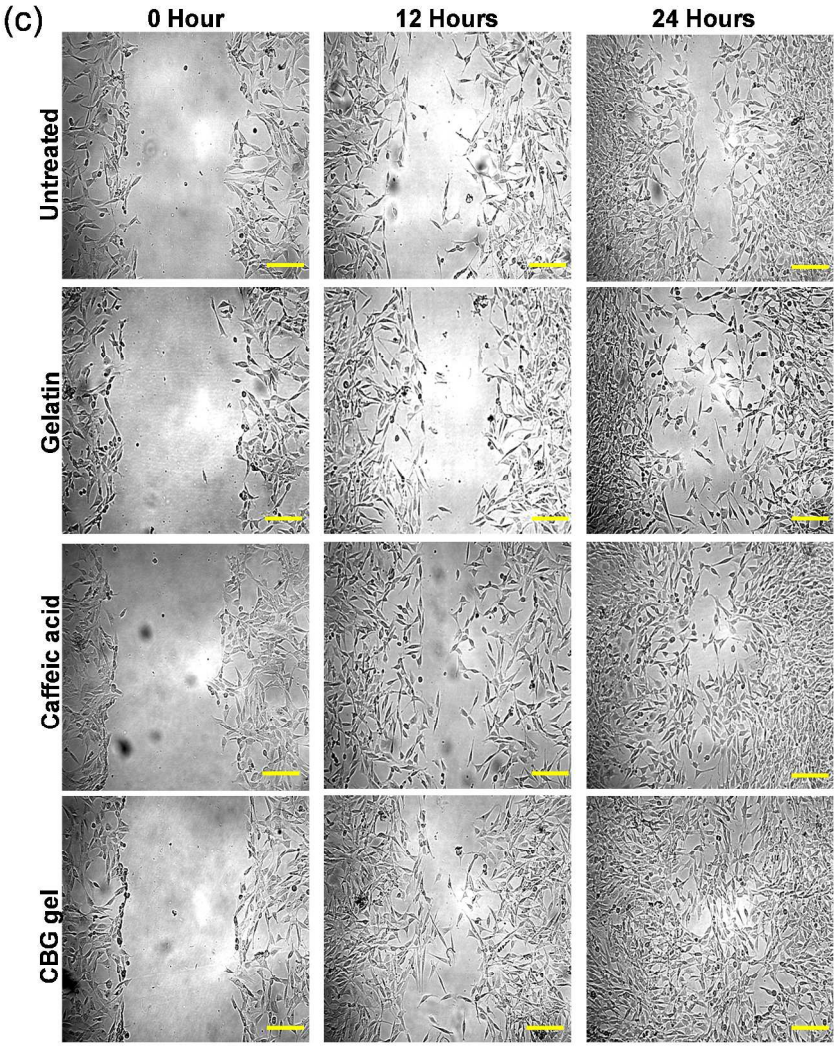


Fig. 3





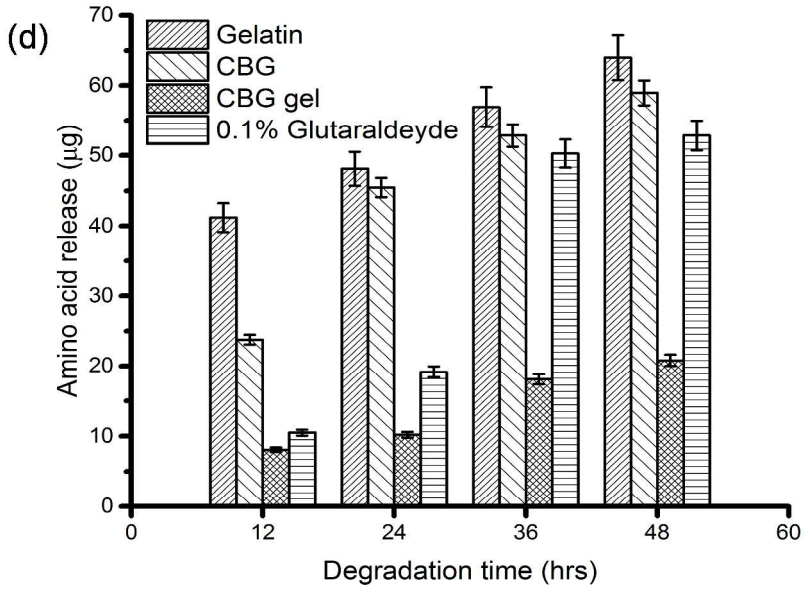



Fig. 4a

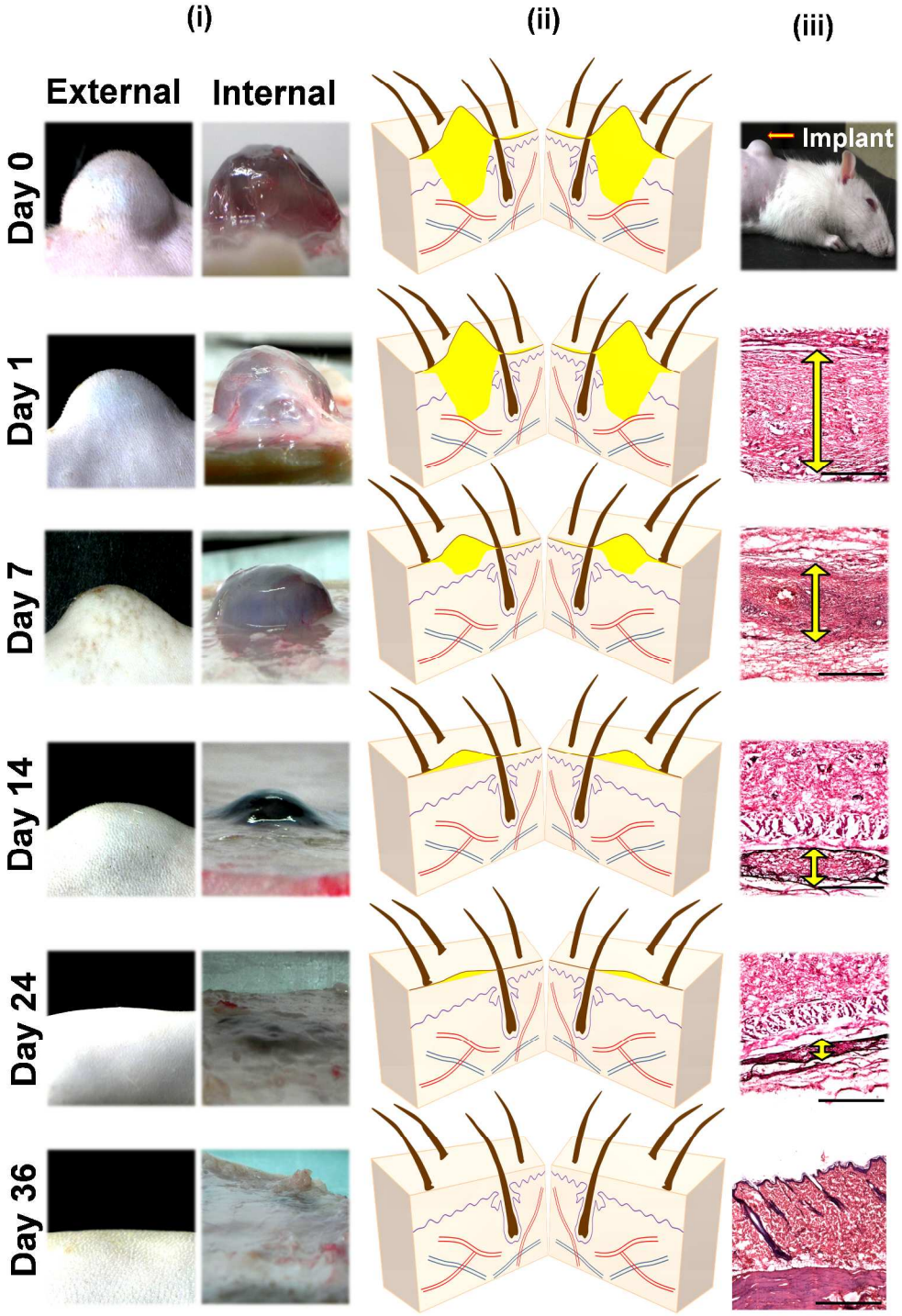


Fig. 4b

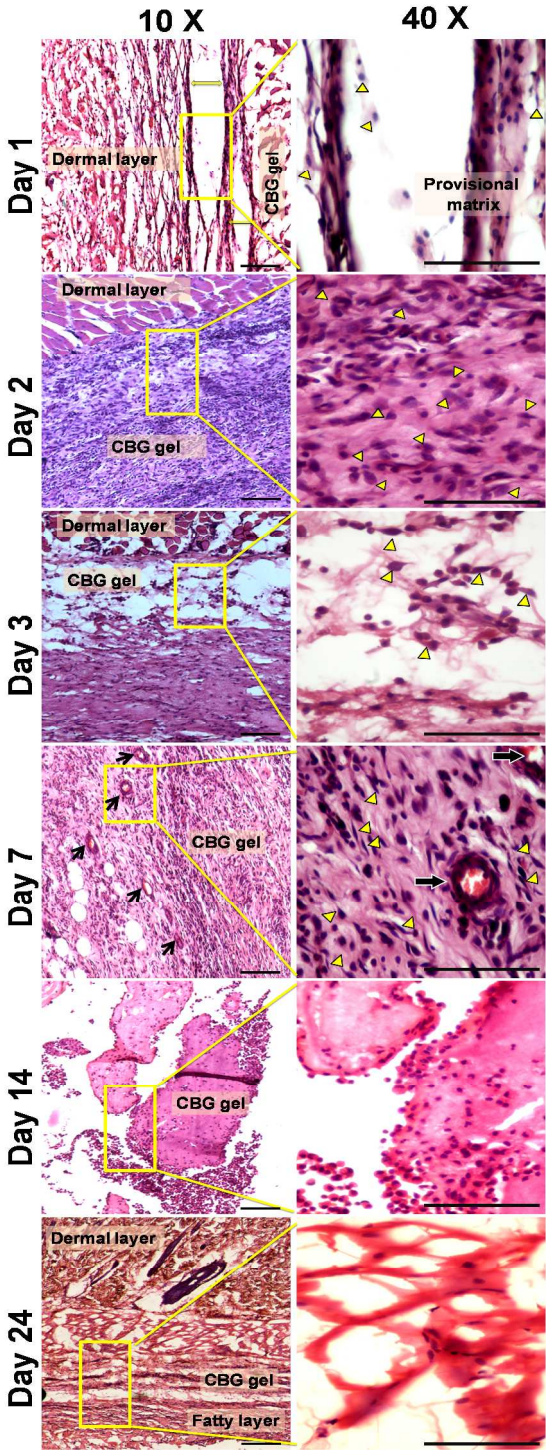
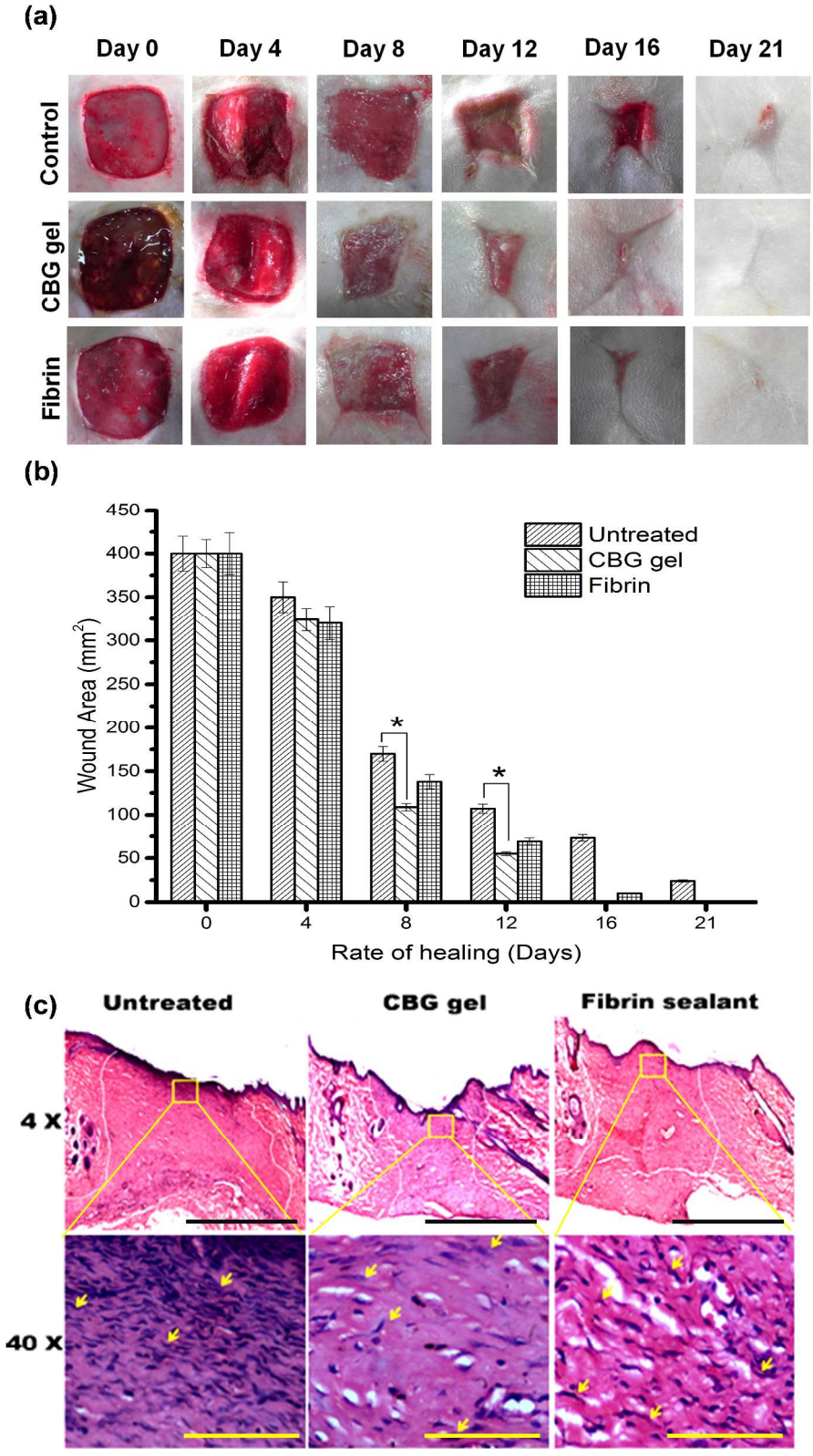


Fig. 5



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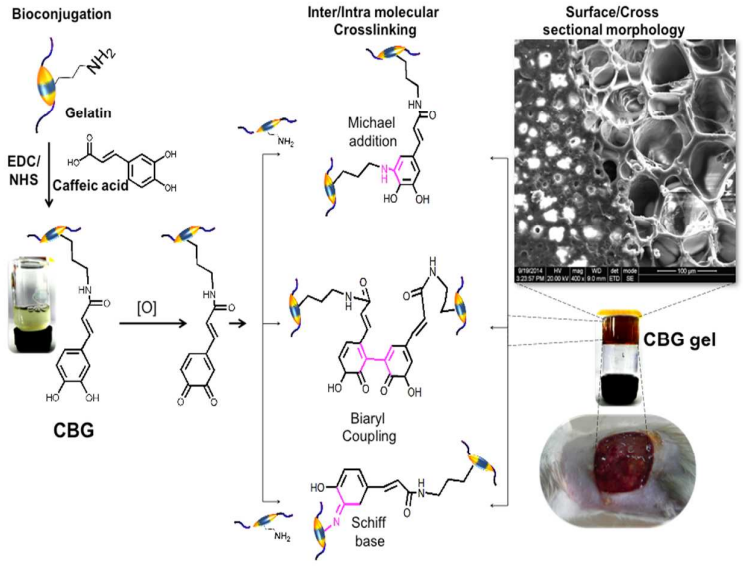


A novel bioconjugated injectable hydrogel with multifunctional properties for wound healing and drug delivery: a biomimic adhesive material